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ATTORNEY'S DOCKET NO. 24679

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U.S. DEPARTMENT OF COMMERCE, PATENT AND TRADEMARK OFFICE		DATE: 18 June 2001 (18.06.2001)
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371		U.S. APPLICATION NO. (if known): Not Yet Assigned 09/0868328
INTERNATIONAL APPLICATION NO.: PCT/KR00/01183	INTERNATIONAL FILING DATE: 19 October 2000 (19.10.00)	PRIORITY DATE CLAIMED: 19 October 1999 (19.10.99)
TITLE OF INVENTION: ENZYMATIC PRODUCTION OF DIFRUCTOSE DIANHYDRIDE IV FROM SUCROSE AND RELEVANT ENZYMES AND GENES CODING FOR THEM		
APPLICANT(S) FOR DO/EO/US: 1) RHEE, Sangki; 2) SONG, Kibang; 3) KIM, Chulho; 4) RYU, Eunja; 5) LEE, Yongbok		
Applicant hereby submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information		
<p>1. <input checked="" type="checkbox"/> This is a FIRST submission of items concerning a filing under 35 U.S.C. 371</p> <p>2. <input type="checkbox"/> This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. 371</p> <p>3. <input type="checkbox"/> This express request to begin national examination procedures (35 USC 371(f)). The submission must include items (5), (6), (9) and (21) indicated below.</p> <p>4. <input type="checkbox"/> A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.</p> <p>5. <input checked="" type="checkbox"/> A copy of the International Application as filed (35 U.S.C. 371(c)(2)):</p> <p style="margin-left: 40px;">a. <input checked="" type="checkbox"/> is transmitted herewith (required only if not transmitted by the International Bureau).</p> <p style="margin-left: 40px;">b. <input type="checkbox"/> has been communicated by the International Bureau.</p> <p style="margin-left: 40px;">c. <input type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US)</p> <p>6. <input type="checkbox"/> A English translation of the International Application as filed (35 U.S.C. 371(c)(2)).</p> <p>7. <input checked="" type="checkbox"/> Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))</p> <p style="margin-left: 40px;">a. <input type="checkbox"/> are attached hereto (required only if not transmitted by the International Bureau).</p> <p style="margin-left: 40px;">b. <input type="checkbox"/> have been communicated by the International Bureau.</p> <p style="margin-left: 40px;">c. <input type="checkbox"/> have not been made; however, the time limit for making such amendments has NOT expired.</p> <p style="margin-left: 40px;">d. <input checked="" type="checkbox"/> have not been made and will not be made.</p> <p>8. <input type="checkbox"/> An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).</p> <p>9. <input type="checkbox"/> An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).</p> <p>10. <input type="checkbox"/> A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).</p> <p>ITEMS 11 to 20 BELOW CONCERN OTHER DOCUMENT(S) OR INFORMATION INCLUDED:</p> <p>11. <input type="checkbox"/> An Information Disclosure Statement under 37 CFR 1.97 and 1.98.</p> <p>12. <input type="checkbox"/> An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included</p> <p>13. <input checked="" type="checkbox"/> A FIRST preliminary amendment.</p> <p>14. <input type="checkbox"/> A SECOND or SUBSEQUENT preliminary amendment</p> <p>15. <input type="checkbox"/> A substitute specification</p> <p>16. <input type="checkbox"/> A change of power of attorney and/or address letter.</p> <p>17. <input type="checkbox"/> A computer-readable form of the sequence listing in accordance with PCT Rule 13ter2 and 35 USC 1821 - 1825</p> <p>18. <input type="checkbox"/> A second copy of the published international application under 35 USC 154(d)(4)</p> <p>19. <input type="checkbox"/> A second copy of the English language translation of the international application under 35 USC 154(d)(4)</p> <p>20. <input checked="" type="checkbox"/> Other items or information:</p> <p>TRANSMITTAL FORM; FEE CALCULATION; INTERNATIONAL PUBLICATION WO 01/29185 A1 WITH; INTERNATIONAL PUBLICATION DATE 26 APRIL 2001 CONSISTING OF 68 PAGES INCLUDING; 45 PAGES TEXTUAL SPECIFICATION, 1 PAGE RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT; 6 PAGES OF 24 CLAIMS, 1 COVER SHEET CONTAINING THE ABSTRACT; 9 SHEETS DRAWINGS; 6 PAGES SEQUENCE LISTING; PRELIMINARY AMENDMENT WITH CLEAN COPY, UNEXECUTED INVENTOR'S DECLARATION; PCT/ISA/220 NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL SEARCH REPORT OR DECLARATION; PCT/ISA/210 INTERNATIONAL SEARCH REPORT; UNEXECUTED INVENTOR'S DECLARATION; PCT/RO/101 REQUEST; PCT/IB/301 NOTIFICATION OF RECEIPT OF RECORD COPY</p>		

ATTORNEY'S DOCKET NO: 24679

<p>09/868328 U.S. APPLICATION NO. (if known) not yet assigned</p>	<p>INTERNATIONAL APPLICATION NO. PCT/KR00/01183</p>	<p>DATE: 18 June 2001 (18 06 2001)</p>
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a. ☒ One check in the amount of \$616.00 to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. 14-0112 in the amount of \$_____ to cover the above fees. (A duplicate copy of this sheet is enclosed.)

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 14-0112.

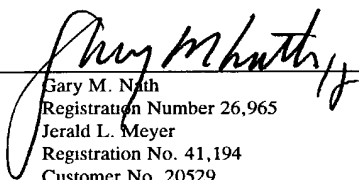
d. Fees are to be charged to a credit card ____ WARNING: Information on this form may become public ____ Credit Card Information should not be included on this form. ____ Provide credit card information and authorization on PTO-2038 ____

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed to request that the application be restored to pending status.

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09868328

BOX PCT

Attorney Docket No. 24679

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

JC03 Rec'd PCT/PTC

18 JUN 2001

1)RHEE, Sangki; 2)SONG, Kibang 3)KIM, Chulho; 4)RYU, Eunja;
5)LEE, Yongbok

International Application No. PCT/KR00/01183

Serial No. NOT YET ASSIGNED

International Filing Date: 19 October 2000 (19 October 2000)

Filed: June _____, 2001

For: **ENZYMATIC PRODUCTION OF DIFRUCTOSE DIANHYDRIDE IV FROM
SUCROSE AND RELEVANT ENZYMES AND GENES CODING FOR THEM**

PRELIMINARY AMENDMENT

Commissioner for Patents
Washington, D.C. 20231

Sir:

Prior to examining on the merits and calculating the filing fee for the application filed herewith, please enter the following amendments:

IN THE SPECIFICATION:

Please amend the specification at page 6, line 3 by deleting the sequence through line 26 and inserting the sequence entitled "novel levansucrase gene of *zymomonas mobilis*", as listed on the attachment 1 with this preliminary amendment.

Please amend the specification on page 26, by deleting line 16, the wording of "TABLE 2.roductionP Amounts of Levan According to pH of Buffer(g/l)" as shown on attachment 2 with this preliminary.

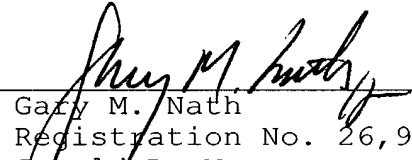
REMARKS

The above amendments have been made to correct clerical mistakes that were not amended during the international phase. No new matter has been added. Pursuant to the new rules implementing the AIPA, a clean copy of the changes to the specification are attached.

Respectfully submitted,

NATH & ASSOCIATES PLLC

By: _____


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Date: June 18, 2001
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GMN/LCH/JLM/dd:AMENDpreml.AIPA

Difructose dianhydride was first found in 1929 by Jackson et al. by analyzing a by-product which was produced when inulin was treated with sulfuric acid to prepare a fructose syrup. Difructose dianhydride, a kind of a cyclic disaccharide, consists of two fructose residues in which a reducing end of each residue is linked to a non-reducing hydroxy group of the counter residue. There have been discovered five kinds of difructose dianhydride, named DFA I to DFA V, thus far. Of them, DFA II and V are found to be synthesized only chemically while the others can be produced enzymatically: DFA I and III are produced from inulin by the action of inulin fructotransferase; and DFA IV from levan by the action of levan fructotransferase.

Difructose dianhydride is a non-digestive, non-fermentative sub-saccharide which is not digested in the animal body. In addition to being useful as a low-calorie sweetener, the sub-saccharide plays a role in inhibiting tooth decay and as a productive factor for *Bifidus* bacteria. It is also reported that
5 difructose dianhydride is used as an absorption factor of minerals in the body (Baik, B. H, Lee, Y. W., and Lee, Y. B.; U.S. Pat. No. 5,700,832, UK. Pat. No. GB 2 308 547 A, Japanese Pat. Appl'n No. 8-51370, Korean Pat. Laid-Open Publication No. 96-13376, Sakurai et al., 1997).

Representative of polyfructans, inulin and levan, both naturally occurring
10 fructose homopolysaccharides, are used to prepare difructose dianhydride (Han, 1989). From them, difructose dianhydride can be synthesized chemically or enzymatically. Chemical synthesis of difructose dianhydride from the natural polyfructans, however, suffers from significant disadvantages. For example, chemical synthesis is of low reaction selectivity, followed by complicated
15 separation and purification. What is worse, it produces pollution of the environment. Consequently, these problems do not vest economical production value in the chemical synthesis. In contrast, enzymatic synthesis using biocatalysts, such as microbes or enzymes, is now regarded as being very economically favorable in synthesizing difructose dianhydride from the natural
20 polyfructans.

Since the discovery of difructose dianhydride III synthase (inulin fructotransferase) from microbes by Tanaka in 1972, enzymes that have the function of synthesizing difructose dianhydrides have been isolated from several microbial sources. The difructose dianhydrides which can be synthesized by
25 such microbe-derived enzymes include DFA I, III and IV. DFA IV is synthesized from levan by the catalytic action of levan fructotransferase and two microorganisms, *Arthrobacter ureafaciens* and *Arthrobacter nicotinovorans* GS-

9 are found to produce DFA IV.

Only a very small amount of levan fructotransferase is synthesized from these bacteria and, thus, its use in the production of difructose dianhydride is very unfavorable in terms of technical and economical aspects. Now generally,
 5 in order to obtain a large amount of a gene of interest, a gene recombinant technique is employed. That is, a levan fructotransferase gene is first isolated from its microbial source and cloned, followed by mass-expression in *E. coli*. Of the levan fructotransferase-producing strains discovered so far, only *A. nicotinovorans* GS-9 is achieved in cloning its levan fructotransferase gene.

10 Being a substrate of levan fructotransferase to produce DFA IV, levan, a homopolysaccharide of fructose, is prepared from sucrose by the transfructosylation reaction of levansucrase. The enzymes which can catalyze the hydrolysis of sucrose are exemplified by sucrase (beta-D-fructofuranosidase, EC 3.2.1.26), levanase (beta-2,6-D-fructan fructanohydrolase, EC 3.2.1.65),
 15 levansucrase (beta-2,6-fructan: D-glucose-1-fructosyl transferase, EC 2.4.1.10), maltase (alpha-D-glucoside glucohydrolase, EC 3.2.1.20), etc. Levan can be produced from sucrose by taking advantage of the transfructosylation activity of levansucrase (Tanaka & Yamamoto, *J. Biochem.*, 85, 287 (1979)).

There are disclosed methods for the production of levan using
 20 levansucrase. For example, it is described in U.S. Pat. No. 4,879,228 and International Publication No. WO 86-4091 that levan is produced by a fermentation process in which advantage is taken of the microbes employing sucrose in their metabolism. The patents, however, suffer from many problems. There is required a lengthy culture period to produce levan.
 25 Further, because the culture contains various products, a difficult purification procedure is needed, giving rise to a decrease in the production yield of levan. When account is taken of these problems, an enzyme reaction process has an

advantage over the culture process in that, because the products of levansucrase are dependent on the reaction conditions for the production of levan from sucrose, desirable products can be obtained by controlling the conditions with ease.

5 It is reported that levan finds numerous applications in the medicinal field, such as a serum substituent (Dedonder *et al.*, *Bull. Soc. Chim. Biol.*, 39, 438 (1957); Schechter *et al.*, *J. Lab. Clin. Med.*, 61, 962(1963)), a colloid stabilizer, an immune agent, a pharmacological enhancer, etc. (Leibovici *et al.*, *Anticancer Res.*, 5, 553 (1985); Stark *et al.*, *Br. J. Exp. Path.*, 67, 141 (1986)),
10 the foodstuffs field, such as quality improver, a stabilizer, an additive for health food, etc. (Hatcher *et al.*, *Bioprocess, Technol.*, 11, 1(1989)), the typographic field, and the cosmetic field (Whiting *et al.*, *J. Inst. Brew.*, 73, 422 (1967); Han, *Bioprocess Technol.*, 12, 1(1920)).

15 DISCLOSURE OF THE INVENTION

It is an object of the present invention to provide an enzymatic process for producing levan from sugar at a high yield.

It is another object of the present invention to provide a levansucrase gene derived from *Z. mobilis*, which contains such a specific tool as to allow its
20 protein to be purified with ease.

It is a further object of the present invention to provide a recombinant expression vector carrying a levansucrase gene derived from *Z. mobilis*, which can express levansucrase in *E. coli*.

It is still a further object of the present invention to provide a bacterial
25 species which is transformed by the recombinant expression vector and can produce levansucrase.

It is yet a further object of the present invention to provide a process of preparing levansucrase by taking advantage of the transformed bacteria.

It is still another object of the present invention to provide an enzymatic process for producing DFA IV from levan at a high yield.

5 It is yet another object of the present invention to provide a novel bacteria *A. ureafaciens* K2032, which produces levan fructotransferase.

It is yet another object of the present invention to provide a novel gene coding for levan fructotransferase, derived from *A. ureafaciens* K2032.

10 It is yet another object of the present invention to provide a recombinant levan fructotransferase gene, which contains such a specific tool as to allow its protein to be purified with ease.

It is yet another object of the present invention to provide a recombinant expression vector carrying the recombinant gene and a bacterial species which anchors the recombinant expression vector.

15 It is yet another object of the present invention to provide a process of preparing levan fructotransferase by taking advantage of the transformed bacteria.

20 It is yet another object of the present invention to provide an enzymatic process for producing DFA IV from sucrose at a high yield by utilizing the levansucrase and the levan fructotransferase.

In accordance with a first embodiment of the present invention, there is provided a process for producing levan from sugar, in which a sugar solution is subjected to reaction at room temperature or lower in an acidic buffer of pH 3.0-7.0 in the presence of a levansucrase derived from *Z. mobilis*. In one version of
25 this embodiment, the reaction is carried out at 0-15 °C and the sugar solution has a sugar concentration of 10-30 % (w/v).

In accordance with a second embodiment of the present invention,

there is provided a novel levansucrase gene of *Z. mobilis*, which has the following base sequence:

```

1  GCGGTGCACCCGACTTCCCTCGACGACCACCGTCCCTACCGGCCGACCGCCGGCCGACTGCTCCTCAGCCTAGACGGGCCCTC
5  91  CTCGAGGTCTTCGTGGGGACGGTGAGGGGACTGCGTCGAACCTGGTCTCTCTGGGGGCCGGCGGTGTGACCGCGAGCCTCGAGACGGCA
181 CGGCCAGGAACCGTGACGTGACCGGATCGACGTGAGGGCGCCAGCGATGCTGACGCCCTGAACCTGCCCGCTTCTGGGCTGACGA
271 GCGTCCCAACCCGACAGCTCTCTTACCGCTGCCGAACAGGGTGGACGCTTCTGCGGCCACCCGTCCACGAGAGGAACAGCA
361 ATGACGCGCGCCATCTCACGCCGCGCGGTGCTCCAGGGAGCGCGCGCCGAGCACTCGCCCTGATCTTCGGCGGTGTGTGCCGCTGCA
451 GCCCGGGCATCCGCTCCGGGCTCGTCCGTGCCGTCTACCATGACGCCCCCAGCGGCTGGCTCTGCGACCCCCAACGCCCGGTCAAC
10 541 ACCCAGCGCGCTACCAAGCTGTACTACCTGCACTCCGACCAGAACAACGGCCCCGGCGGTGGGACCACGCGAGCACGACCGACGGCGTC
631 GCCTTCACGCAACACGGCACCGTGATGCCGTGCGGCCGACTTCCCCGTGTGGTCCGGGTGCGCGGTCTGCGCACCCGCAACACGGCA
721 GGGTTCGGCGCCGCGCGGTGCTCGCGCTCGCGACCCAGCCGACCGACGGCGTCCGCAAGTACCAGGAGCAGTACCTCTACTGGTCGACC
811 GACGGCGGGTTCAGTTCAACGCCCTGCCCGACCCCGTCATCGTCAACACCGACGGTCGCGCCGCCACACGCCCGCGGAGATCGAGAAC
901 GCCGAGTGGTTCGCGACCCCAAGATCCACTGGGACACCGCCCGCGAGAATGGGTCTGCGTCATCGGACGACTGCGGTACGCCCGGTTT
15 991 TACACCTCGCGAACCTGCGCGACTGGACACTTCGCCGCAACTTCGACTACCCGAACACGCCCTCGCGCGCATCGAGTGCCCCGACCTG
1081 TTCGAGATCACCGCAGACGACGGGACACGCCACTGGGTGCTCGCCGCCAGCATGGACGCCCTACGGCATCGGCTCCCATGACGTACGCC
1171 TACTGGACAGGCACCTGGGACGCGGAGCAGTTCCACGCCGACGACCTACCCCGCAATGGTTCGACTGGGGCTGGGACTGGTACGCGGCC
1261 GTCACTTGGCCATCGATCGACGCGCCGAGACCAAGCGCTCGCCATCGCGTGGATGAACAACTGGAAGTACGCGCACGCGACGTCCCC
1351 ACCGACGCATCCGACGGCTACAACGGGCAGAACTCGATCGTCCGCGAGCTGCGGCTCGCCGACAGCCTGGCGGTGGTACACCTCTCTG
20 1441 AGCACCCCCGTGGCAGCGCTGACGAACTACGTACCGCCACCAACCACTCCCCGACCGACCGTCGACGGCAGCGCCGCTCTGCCATGG
1531 AACGGACGCGCATACGAGATCGAGCTCGACATCGCTGGGACACCGCGACGAACGTCGGCATCTCGGTGGGCGCTCCCCGACGGAAAC
1621 CGGCACACGAATCGGCAAGTACGGAGCAGACCTGTACGTGACCGAGGACCCTCCGACCTCGCGGGTACTCGCTCGCCCCCTACTCG
1711 CGAGCCGCCGCCCCATCGACCCCGCGCCGATCCGTGACCTCGCATCTCTCGTCGACACCCAGAGCGTCGAGGTCTCTGTCAACGCC
1801 GGCCACACCGTGTCTCCAGCAGGTCCACTTCGCCGAGGGCGACACGGGAATCTCGCTTACACCGACGGCGGCCCGCACACTTCACC
25 1891 GGCATCGTCGTCGCGAGATTGGCCAGGCGATCTAGCGATGCACACACCGCTCACCGAAGCCGCGCCCGGGAGACGACGGCCGAC
1981 AATCGACACGTCTCTGTCGTT

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In accordance with a third embodiment of the present invention, there is provided a levansucrase of *Z. mobilis*, which has the following amino acid sequence:

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1  M L N K A G I A E P S L W T R A D A M K V H T D D P T A T M
31 P T I D Y D F P V M T D K Y W V W D T W P L R D I N G Q V V

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61 S F Q G W S V I F A L V A D R T K Y G W H N R N D G A R I G
 91 Y F Y S R G G S N W I F G G H L L K D G A N P R S W E W S G
 121 C T I M A P G T A N S V E V F F T S V N D T P S E S V P A Q
 151 C K G Y I Y A D D K S V W F D G F D K V T D L F Q A D G L Y
 5 181 Y A D Y A E N N F W D F R D P H V F I T P K I G K T Y A L F
 211 E G N V A M E R G T V A V G E E E I G P V P P K T E T P D G
 241 A R Y C A A A I G I A Q A L N E A R T E W K L L P P L V T A
 271 F G V N D Q T E R P H V V F Q N G L T Y L F T I S H H S T Y
 301 A D G L S G P D G V Y G F V S E N G I F G P Y E P L N G S G
 10 331 L V L G N P S S Q P Y Q A Y S H Y V M T N G L V T S F I D T
 361 I P S S D P N V Y R Y G G T L A P T I K L E L V G H R S F V
 391 T E V K G Y G Y I P P Q I E W L A E D E S S N S A A A L S L
 421 L N K *

15 In accordance with a fourth embodiment of the present invention, there is provided a recombinant expression vector, carrying the levansucrase gene.

In accordance with a fifth embodiment of the present invention, there is provided a novel bacteria *E. coli* BL21 (DE3)/pEL12 (KCTC 8661), in which the plasmid carries a levansucrase gene derived from *Z. mobilis*.

20 In accordance with a sixth embodiment of the present invention, there is provided a process of preparing levansucrase, comprising the steps of culturing a bacterial species anchoring a levansucrase gene-carrying, expression plasmid, harvesting and homogenizing the cells, and isolating levansucrase from the cell homogenate. In one version of this embodiment, the levansucrase gene has a
 25 base sequence stretch encoding histidine residues at its 5'- or 3'-end. In another version of this embodiment, the isolating step is carried out using metal ion-affinity chromatography and the bacterial species is *E. coli* BL21(DE3)/pEL12 (KCTC 8661P).

In accordance with a seventh embodiment of the present invention,
 30 there is provided a novel microorganism *A. ureafaciens* K2032, which shows an

30
1 CGGTGCACCCCGACTTCCTCCCTCGACGACACCCTGCCCTTACCGGCCGACGCCGCCGCCGACTGCTCTCAGCCTAGACGGGCCCTC
91 CTCGAGGTCTTCGTCGGGGACGGTGAGGCGACTGCGTCGAACCTGGTCTCTCTGGGGCCGGCGGTGTGACCGCGAGCCTCGAGACGGCA
181 CGGCCAGGAACCGTGACAGTGAACCGCATGACGTCGAGGCGCCACGCGATGCTGACGCCCTGAACTGCCGCCCTTCTGGGCTGACGA
271 GCGCTCCACCCCGACAGCTCTCTTCTACCGCTGCCCGAACACGGGTGGACGCTTCTGTCGCCCCACCCGTCCACGAGAGGAACACGCA
361 ATGACGCCGCCCATCTCACGCCGCCCGTGCTCCAGGAGGCCGCCGCCGAGCACTCGCCCTGATCTTCGGCGGTGTGTGCCCTGCA

451 GCCCGGGCATCCGCTCCGGGCTCGCTCCGTGCCGTCTACCATGACGCCCCCAGCGGCTGGCTCTGCGACCCCCAACGCCCGGTACCC
5 541 ACCCACGGCGCTACCAGCTGTACTACCTGCACTCCGACCAGAACAACGGCCCCGGCGGCTGGGACCACGCGAGCAGACCCGACGGCGTC
631 GCCTTCACGCACACGGCACCGTGATGCCGCTGGGCCCCGACTTCCCCGTGTGGTCCGGGTCGGCGGTCGTGCGACCCGGAACACGGCA
721 GGGTTCGGCGCGCGCGGTCGTGCGCTCGCGACCCAGCCGACCGACGGCGTCCGCAAGTACCAGGAGCAGTACTCTACTGGTCGACC
811 GACGGCGGGTTACAGTTACCCGCCCTGCCCGACCCCGTCATCGTCAACACCGACGGTCGCGCCGCCACCACGCCCGCGAGATCGAGAAC
901 GCCGAGTGGTTCGCGACCCCAAGATCCACTGGGACACCGCCCGCGGAGAATGGGTCTGCGTCATCGGACGACTGCGGTACGCCCGGTTC
991 TACACCTCGCCGAACCTGCGGACTGGACACTTCGCCGCAACTTCGACTACCCGAACACGCCCTCGGCGGATCGAGTGCCCGGACCTG
1081 TTCGAGATCACCGCAGACGACGGGACACGCCACTGGGTGCTCGCCGCCAGCATGGACGCCCTACGGCATCGGCTCCCCATGACGTACGCC
1171 TACTGGACAGGCACCTGGGACGGCGAGCAGTTCCAGCCGACGACCTACCCCGCAATGGCTCGACTGGGGCTGGGACTGGTACGCGGCC
1261 GTCACCTGGCCATCGATCGACGCGCCGAGACCAAGCGCTCGCCATCGCGTGGATGAACAACTGGAAGTACGCGCAGCGGACGTCCCC
1351 ACCGACGCATCCGACGGCTACAACGGGAGAACTCGATCGTCCGCGAGCTGCGGCTCGCCGACAGCCTGGCGGCTGGTACACCTCTCTG
1441 AGCACCCCGTGGCAGCGCTGACGAATACGTACCGCCACCAACCACTCCCCGACCGGACCGTCGACGGCAGCGCGCTCTGCCATGG
1531 AACGGACGCGCATACGAGATCGAGCTCGACATCGCTGGGACACCGCGACGAACGTGCGCATCTCGGTGGGCGCTCCCCGACGGAACC
1621 CGGCACACGAACATCGGCAAGTACGGAGCAGACCTGTACGTGACCGAGGACCCTCCGACCTCGCCGGTACTCGCTCGCCCCCTACTCG
1711 CGAGCGCGCGCCCATCGACCCCGCGCGCCGATCCGTGACCTGCGCATCTCTGTCGACACCCAGAGCGTCGAGGTCTTCGTCAACGCC
1801 GGCCACACCGTGTCTCCAGCAGGTCCACTTCGCCGAGGGGACACGGGAATCTCGCTCTACCCGACGGCGGCCCGCACACTTCACC
1891 GGCATCGTCTCGCGAGATTGGCCAGGCGATCTAGGCGATGCACACCACACCGCTACCGAAGCCGCGCCCGGGAGACGACGGCCGAC
1981 AATCGACACGTCTCTCGTCGTT

20 In accordance with a tenth embodiment of the present invention, there is
provided a novel levan fructotransferase gene of sequence 3:

1 GCGGTGACCCCCGACTTCCCTCGACGACCACCGTCCCCCTACCGGCGGACCGCCCGGCGGACTGCTCCTCAGCCTAGACGGGCCCTC
AvaI
25 91 CTCGAGGTCTTCGTGCGGGACGGTGAGGCGACTGCGTCGAACCTGGTCTCTCTGGGGGCGGCGGTGTGACCGCGAGCCTCGAGACGGCA
181 CGGCCAGGAACCGTGACGTGACCGGATCGACGTGAGGCGGCCAGCGATGCTGACGCCCCGAACTGCCGCGTTCTGGGCTGACGA
271 GCGTCCCAACCCGACAGCTCTCCTTCTACCGCTGCCCGAACAGGGTGGACGCTTCGTGCGGCCACCCGTCCACGAGAGGAACACGAC
PstI
361 ATGACGCGCGCATCTCAGCGCGCGCGTGTCTCAGGGAGCGCGCGCGGAGCACTCGCCCTGATCTTCGCGGCTGTGTGCCGCTGCA
30 1 M T P A I S R R A V L Q G A G A G A L A L I F G G A V P P A
451 GCCCGGGCATCCGCTCCGGGCTCGTCCGTGCCGTCTACCATGACGCCCCCAGCGGCTGGCTCTGCGACCCCCAACGCCCGGTACCC
31 A R A S A P G S L R A V Y H M T P P S G W L C D P Q R P V T
541 ACCCACGGCGCTACCAGCTGTACTACCTGCACTCCGACCAGAACAACGGCCCCGGCGGCTGGGACCACGCGAGCAGACCCGACGGCGTC
61 T H G A Y Q L Y Y L H S D Q N N G P G G W D H A S T T D G V

631 GCCTTCACGCACCACGGCACCGTGATGCCGCTGCGGCCGACTTCCCCGTGTGGTCCGGGTCGGCGGTCTCGGCACCGCGAACACGGCA
91 A F T H H G T V M P L R P D F P V W S G S A V V G T A N T A
721 GGGTTCGGCGCCGGCGCGGTCTGTCGCTCGCGACCCAGCCGACCGAGCGGTCCGCAAGTACCAGGAGCAGTACCTCTACTGGTCGACC
121 G F G A G A V V A L A T Q P T D G V R K Y Q E Q Y L Y W S T
5 811 GACGGCGGGTTCACGTTCACCGCCCTGCCCGACCCCGTCATCGTCAACACCGACGGTCGCGCCGCCACACGCCCGCGAGATCGAGAAC
151 D G G F T F T A L P D P V I V N T D G R A A T T P A E I E N
901 GCCGAGTGGTTCGCGACCCCAAGATCCACTGGGACACCGCCCGCGGAGAATGGGTCTCGCTCATCGGACGACTGCGGTACGCCCGCTTC
181 A E W F R D P K I H W D T A R G E W V C V I G R L R Y A A F
991 TACACCTCGCCGAACCTGCGGACTGGACACTTCGCCGCAACTTCGACTACCCGAACACGCCCTCGGCGGCATCGAGTGCCCGGACCTG
10 211 Y T S P N L R D W T L R R N F D Y P N H A L G G I E C P D L
1081 TTCGAGATCACCGCAGACGACGGGACACGCCACTGGGTGCTCGCCGCCAGCATGGACGCCCTACGGCATCGGCCTCCCATGACGTACGCC
241 F E I T A D D G T R H W V L A A S M D A Y G I G L P M T Y A
1171 TACTGGACAGGCACCTGGGACGGCGAGCAGTTCACGCCGACGACCTACCCCGCAATGGCTCGACTGGGGCTGGGACTGGTACGCGGGCC
271 Y W T G T W D G E Q F H A D D L T P Q W L D W G W D W Y A A
15
Clal Aval
1261 GTCACTTGCCATCGATCGACGCGCCCGAGACCAAGCGCTCGCCATCGCTGGATGAACAACTGGAAGTACGCCGCACGCGACGTCCCC
301 V T W P S I D A P E T K R L A I A W M N N W K Y A A R D V P
1351 ACCGACGCATCCGACGGCTACAACGGGCAGAACTCGATCGTCCGCGAGCTGCGGCTCGCCCGACAGCCTGGCGGTGGTACACCTCTCTG
331 T D A S D G Y N G Q N S I V R E L R L A R Q P G G W Y T L L
20
NcoI
1441 AGCACCCCGTGGCAGCGCTGACGAACTACGTACCGCCACCAACCACTCCCCGACCGGACCGTCGACGGCAGCGCCGCTCTGCCATGG
361 S T P V A A L T N Y V T A T T T L P D R T V D G S A V L P W
1531 AACGGACGCGCATACGAGATCGAGCTCGACATCGCTGGGACACCGGACGAACTGCGCATCTCGGTGGGCGCTCCCCGACGGAACC
391 N G R A Y E I E L D I A W D T A T N V G I S V G R S P D G T
25 1621 CGGCACACGAACATCGGCAAGTACGGAGCAGACCTGTACGTTCGACCGAGGACCCTCCGACCTCGCCGGTACTCGCTCGCCCCCTACTCG
421 R H T N I G K Y G A D L Y V D R G P S D L A G Y S L A P Y S
1711 CGAGCCGCGCCCCCATCGACCCCGGCGCCGATCCGTGCACCTGCGCATCTCTCGTCGACACCCAGAGCGTCGAGGTCTTCGTCAACGCC
451 R A A A P I D P G A R S V H L R I L V D T Q S V E V F V N A
1801 GGCCACACCGTGCTCTCCAGCAGGTCCACTTCGCCGAGGGGACACGGGAATCTCGCTCTACACCGACGGCGGCCCGCACACTTCACC
30 481 G H T V L S Q Q V H F A E G D T G I S L Y T D G G P A H F T
SmaI
1891 GGCATCGTCGTCGCGAGATTGGCCAGGCGATCTAGGCGATGCACACCACACCGCTCACCGAAGCCGCGCCCCGGGAGACGACGCGCGAC
511 G I V V R E I G Q A I *
1981 AATCGACACGTCTCTCGTCGT

In accordance with an eleventh embodiment of the present invention, there is provided a recombinant expression vector, carrying the levan fructotransferase gene.

In accordance with a twelfth embodiment of the present invention, there is provided a process for producing DFA IV from levan, in which a levan solution is subjected to reaction at 25-50°C for 3-10 hours in an acidic buffer of pH 3.0-7.0 in the presence of a levan fructotransferase. In one version of this embodiment, the reaction is carried out at 37°C. In another version of this embodiment, the acidic buffer is a phosphate buffer of pH 5.8 and the levan solution has a levan concentration of 5-15 % (w/v).

In accordance with a thirteenth embodiment of the present invention, there is provided a process of preparing levan fructotransferase, comprising the steps of culturing a bacterial species anchoring a levan fructotransferase gene-carrying, expression plasmid, harvesting and homogenizing the cells, and isolating levan fructotransferase from the cell homogenate. In one version of this embodiment, the levan fructotransferase has histidine residues at its N- or C-terminus and the isolating step is carried out using metal ion-affinity chromatography.

In accordance with an fourteenth embodiment of the present invention, there is provided a process for producing difructose dianhydride IV from sucrose, comprising the steps of reacting a sugar solution at room temperature or lower in an acidic buffer of pH 3.0-7.0 in the presence of a levansucrase to produce levan, purifying the levan from the sugar reaction mixture, partially or completely, reacting a levan solution at 25-50 °C for 3-10 hours in an acidic buffer of pH 3.0-7.0 in the presence of a levan fructotransferase to produce difructose dianhydride IV, and isolating the difructose dianhydride IV from the levan reaction mixture.

BRIEF DESCRIPTION OF THE DRAWINGS

The above and other objects, features and other advantages of the present invention will be more clearly understood from the following detailed description taken in conjunction with the accompanying drawings, in which:

5 Fig. 1 shows the various steps in a process for the production of difructose dianhydride IV from sucrose.

Fig. 2 shows the restriction map of plasmid pZL8, in which B stand for *Bam*HI, H for *Hind*III, E for *Eco*RV, N for *Nco*I, A for *Ase*I, S for *Sph*I, (B::Sa) for *Bam*HI::*Sau*3A1.

10 Fig. 3 shows a base sequence of the levansucrase gene and an amino acid sequence deduced therefrom.

Fig. 4 shows a SDS-PAGE result of purified levansucrase, in which a standard protein is electrophoresed in lane M, total protein of *E. coli* in lane 1 and the purified levansucrase in lane 2.

15 Fig. 5 shows a SDS-PAGE result of purified levan fructotransferase, in which a standard protein is electrophoresed in lane 1 and the purified levan fructotransferase in lane 2.

Fig. 6 shows a base sequence of the levan fructotransferase gene and an amino acid sequence deduced therefrom.

20 Fig. 7 shows a structure of the recombinant expression vector pUDFA18 carrying the levan fructotransferase gene, in which B stand for *Bam*HI, P for *Pst*I, N for *Nco*I, S for *Sal*I, C for *Cla*I, and (N/A) for *Nco*I/*Afl*III.

Fig. 8 shows the total protein pattern of *E. coli* JUD81 in SDS-PAGE in which a bacterial protein is electrophoresed in lane 1 and a standard protein in
25 lane 2.

Fig. 9 shows the change of the amounts of DFA IV and other

saccharides in the reaction mixture in accordance with the reaction time period.

Fig. 10 is a NMR result for the DFA IV purified.

BEST MODE FOR CARRYING OUT THE INVENTION

5 The present invention pertains to the enzymatic production of DFA IV from sucrose, which is achieved by carrying out the following process steps: a levan production process by levansucrase, a purification and concentration process of levan, a DFA IV production process by levan fructotransferase, and a DFA IV recovery process by crystallization. Below, a description will be given, in
10 detail, of these processes (Fig. 1).

1. Levan Production Process by Levansucrase

In accordance with the present invention, the production of levan is based
15 on the catalysis of the levansucrase isolated from *Z. mobilis* with sucrose serving as a substrate. In the presence of the levansucrase, sucrose is subjected to reaction at room temperature, preferably at 0-15°C and more preferably at 10°C for 20-80 hours and preferably for 20 hours in an acidic buffer solution of pH 3.0-7.0 and preferably of pH 5.0. Therefore, most preferable is to react the
20 sucrose at 10°C for 20 hours in an acetic acid buffer of pH 5.0.

As for the substrate, its examples include refined sugar and raw sugar with preference to refined sugar.

The sugar preferably has a concentration of 10-30 % (w/v) based on the total volume of the culture and most preferably 20 % (w/v).

25 Regardless of whether it is natural or is made by genetic recombination, the levansucrase that is derived from *Zymomonas mobilis* is useful in the present

invention. For the effective catalysis of levan production, the enzyme is used at an amount of 0.42-3.0 U/ml based on the total volume of the reaction solution, and preferably at an amount of 2.08 U/ml.

Usually, gene manipulation leads to the mass production of proteins, which is very advantageous in purification. Thus, the levansucrase of the present invention is preferably obtained through genetic recombination. To this end, first, a plasmid carrying a *Z. mobilis*-derived levansucrase gene, e.g., the plasmid pZL8 obtained from *E. coli* KCTC 8546P, is used as a template to amplify the levansucrase gene by the polymerase chain reaction (PCR) with appropriate synthetic primers. Subsequently, the amplified gene of interest is inserted in a vector which is able to be expressed in a suitable host. For instance, when *E. coli* is used as a host, the levansucrase gene amplified is inserted in pET3d (Stratagene) to construct an *E. coli*-expression plasmid, e.g., pEL11 which is then transformed into the host. This transformed host cell is cultured under an appropriate condition and homogenized, followed by centrifugation. To the supernatant, ammonium sulfate is added to give crude levansucrase.

Since the crude enzyme contains various proteins in addition to levansucrase, a complicated purification procedure is required to obtain pure levansucrase. In order to overcome this problem, there is provided a levansucrase further comprising histidine residues at its N- or C-terminus, which can be simply purified by metal ion-exchange column chromatography, in accordance with the present invention.

Histidine residues can be attached to the N- or C-end of levansucrase as follows. First, PCR is conducted using a set of synthetic primers which are designed to have a histidine base sequence at their 3' or 5'-end while a plasmid carrying a *Z. mobilis*-derived levansucrase gene, e.g., the plasmid pZL8 obtained

2. Purification and Concentration Process of Levan

After completion of the enzymatic production reaction of levan, the
 5 resulting levan-containing solution must be deprived of unreacted residual
 sucrose, glucose, and oligosaccharides, followed by concentration preferably by
 as much as 10-15 %. To this end, available are various techniques, such as
 solvent precipitation, ultrafiltration, diafiltration, reverse osmosis,
 chromatography, etc. For a small scale, such as a laboratory scale, an organic
 10 solvent method using, for example, ethanol is suitable. Only to precipitate
 twice or three times in the organic solvent is sufficient to obtain pure levan.
 For a large scale, a membrane filtration process is preferably employed, as in
 various polymer production processes.

Where the levan is produced in a continuous process, membrane
 15 isolation techniques, such as microfiltration (MF), ultrafiltration (UF) or reverse
 osmosis (RO), are used to effectively isolate the product of interest or remove
 by-products from the reaction solution. These techniques have advantages of
 being able to be operated at low temperatures as well as allow the simple
 measurement of energy yield and separation capacity. For example, the
 20 glucose can be removed from the reaction mixture with the aid of ultrafiltration
 (UHF-500-E-90A, 500,000 molecular weight cutoff, A/G Technology Co.) or
 MF (CPP-1-k-9A, 0.1 microl, A/G Technology co.). Preferred is UHF-500-E-
 90A, which is superior in permeate flux and permeability (permeate flux, 376.47
 ml.ft²; permeability, 21.16 ml/min/ft²/psi; 500,000 nominal molecular weight
 25 cutoff)

To achieve excellent purification of the levan, the filtration described
 above may be used in combination with the organic solvent precipitation.

Alternatively, the purification of the levan may be accomplished by conducting various membrane isolation techniques in multiple steps.

3. DFA IV Production Process by Levan Fructotransferase

5

In accordance with the present invention, DFA IV is produced from partially purified or pure levan by the catalysis of levan fructotransferase.

In the presence of the levan fructotransferase, levan is subjected to reaction at 25-50°C and preferably at 37°C for a time period of 3-10 hours and
10 preferably for 5 hours in a buffer of pH 3.0-7.0 and preferably of pH 5.8. Preferable is a phosphate buffer.

Useful to produce difructose dianhydride is partially or completely purified levan with preference to completely purified levan.

In the reaction solution, the levan is preferably controlled to have a
15 concentration of 5-15 % (w/v) based on the total volume of the culture and most preferably 10 % (w/v).

Regardless of whether it is natural or is made by genetic recombination from transformable microbes such as *E. coli* or yeasts, the levan fructotransferase that is derived from *A. ureafaciens* is useful in the present
20 invention.

The levan fructotransferase of the present invention is preferably obtained through genetic recombination because gene manipulation leads to the mass production of the protein, which is very advantageous in purification. To this end, first, a plasmid carrying an *A. ureafaciens*-derived levan
25 fructotransferase gene, e.g., the plasmid pDA18, is used as a template to amplify the levan fructotransferase gene by PCR with appropriate synthetic primers. Subsequently, the amplified gene of interest is inserted in a vector which is able

to be expressed in a suitable host, such as bacteria or yeast.

In accordance with the present invention, the levan fructotransferase gene amplified is inserted in pUC18 to construct an *E. coli*-expression plasmid pUDFA18, which is then transformed into *E. coli* DH5 α . The new bacterial
5 cell thus obtained was named *E. coli* JUD81 and deposited in Korean Collection for Type Cultures, Korean Research Institute of Bioscience and Biotechnology on Sep. 1, 1999 at accession No. KCTC 8961P. The original deposit was converted to a deposit under the Budapest Treaty on Oct. 19, 2000 at accession No. KCTC 0877BP. This transformed host cell is cultured under an
10 appropriate condition and homogenized, followed by centrifugation. Addition of ammonium sulfate to the supernatant allows the production of crude levan fructotransferase.

Since the crude enzyme contains not only levan fructotransferase, but also various other proteins, the crude enzyme must undergo a complicated
15 purification procedure to obtain only levan fructotransferase. In order to overcome this problem, there is provided a levan fructotransferase further comprising histidine residues at its N- or C-terminus, which can be simply purified by metal ion-exchange column chromatography, in accordance with the present invention.

20 Histidine residues can be attached to the N- or C-end of levan fructotransferase as follows. First, PCR is conducted using a set of synthetic primers which are designed to have a histidine base sequence at their 3' or 5'-end while a plasmid carrying an *A. ureafaciens*-derived levan fructotransferase gene, e.g., the plasmid pUDFA18 obtained from *E. coli* KCTC 0877BP is used
25 as a template, so as to yield a large quantity of a levan fructotransferase gene which further comprises a nucleotide sequence coding histidine at its 3' or 5'-end. Thereafter, the amplified gene is inserted in a vector which is expressable

Isolation of DFA IV from the resulting reaction solution may be

5 In addition to being more stable to heat than sucrose, DFA IV well crystallizes. For the purpose of crystallizing DFA IV, the enzymolyzed solution of levan needs to be concentrated. In this connection, the above-mentioned filtration processes or a popularized standard sugar solution evaporation process may be utilized. Alternatively, the enzymolyzed solution may be primarily concentrated to some degree by filtration and then, heated at 70-80°C until DFA IV reaches a desirable concentration. At this time, a heating temperature exceeding 100 °C causes caramelization. The DFA IV filtrate of the primary filtration may be used as a diluting solution of the levan.

In a small scale site, such as a laboratory, the DFA IV solution is preferably added with two volumes of an organic solvent, such as ethanol, to precipitate oligosaccharides or limited levan and the resulting supernatant is concentrated using a rotary evaporator, after which the concentrate is controlled to an ethanol concentration of 95 % with 100% ethanol and allowed to stand at 4°C to induce crystal precipitation. Alternatively, while a supersaturated solution of DFA IV is allowed to stand at 4°C to induce natural crystallization, DFA IV crystals are seeded to rapidly form crystals. These batch crystallization processes are, however, disadvantageous in that it is difficult to obtain uniform crystals. In contrast, a continuous process is very useful in crystallizing DFA IV at a uniform size and shape as well as has an advantage of being of low cost compared with the batch process.

A better understanding of the present invention may be obtained in light of the following examples which are set forth to illustrate, but are not to be

construed to limit the present invention.

EXAMPLE

5

EXPERIMENTAL EXAMPLE 1

Cloning of Levansucrase Gene

The isolation of genomic DNA from *Z. mobilis* was executed according to the instruction of Raymond and Tate (Raymond and Tate, Recombinant DNA techniques, p.162, 1983, Addison-Wesley Publ.). After being washed with a
 10 TEN buffer (10 mM Tris-Cl pH 7.6, 1 mM EDTA, 10 mM NaCl), 1 g of a mass of *Zymomonas mobilis* ZM1 (ATCC 10988) which had been cultured in a YPS medium (Yeast extract 0.5%, peptone 1%, sucrose 2%) was suspended in 10 ml of an SET buffer (sucrose 20%, 50 mM Tris-Cl pH 7.6, 50 mM EDTA) and
 15 treated with lysozyme (Sigma, 5 mg/ml PEN buffer). After 30 min of the enzyme treatment, the mixture was added with 10 ml of a TEN buffer and 1 ml of 10% sodium dodecylsulfate (SDS) and slowly agitated. To the lysed cells was added 2 ml of a 5 M NaCl solution and 20 ml of a TEN buffer and the resulting solution was added with an equal volume of a phenol solution saturated
 20 with a TE buffer (10 mM Tris-HCl pH 7.6, 1 mM EDTA) and agitated for 5 min, followed by centrifugation at 10,000 rpm for 5 min. The supernatant was further centrifuged after being well mixed with chloroform/n-amyl alcohol (24:1). Addition of two volumes of cold ethanol to the resulting supernatant precipitated DNA.

25

After purification, the *Z. mobilis* genomic DNA was partially cut with the restriction enzyme Sau3AI and DNA fragments in a size range of 4-10 kb were isolated with the aid of 1% agarose gel. Separately, pUC119 (Takara, Japan)

was cut with the restriction enzyme *Bam*HI and dephosphorylated. Recombinant plasmids were obtained by ligating the isolated DNA fragments of 4-10 kb to digested pUC119.

Following the techniques of Mandel and Higa (Mandel and Higa, J. Mol. Biol. 53, 159 (1970)) and Inoue et al. (Inoue et al., Gene 96, 23 (1990)), transformation was executed. First, *E. coli* JM109 that had been cultured at 18°C for 36 hours in 30 ml of an SOB medium (Trypton 2%, Yeast extract 0.5%, 10 mM NaCl, 2.5 mM CaCl₂, 10 mM MgCl₂, 10 mM MgSO₄) was kept in ice for 10 min and centrifuged at 2500xg for 10 min, after which the cell pellet was suspended in 10 ml of a TB solution (10 mM PIPES, 55 mM MnCl₂, 250 mM KCl). The resulting suspension was added with dimethyl sulfoxide (DMSO) to the final concentration of 7 %, kept in ice for 10 min, aliquoted at 400 µl in cold tubes, and stored in a liquid nitrogen tank. For transformation, the frozen cell suspension was thawed at room temperature and well mixed with 10 µl of the recombinant DNA (DNA 1 µg) and this mixture was refrigerated for 30 min and subjected to heat-shock at 42°C for 30 sec. Subsequently, the transformation solution was added with 800 µl of an SOC medium (Trypton 2%, Yeast extract 0.5%, 10 mM NaCl, 2.5 mM CaCl₂, 10 mM MgCl₂, 10 mM MgSO₄, 10 mM Glucose), vigorously shaken at 37°C for 1 hour, spread over the surface of an LB agar plate containing ampicillin and chloramphenicol, and cultured at 37°C for 16 hours.

The colonies formed were transferred to an MG agar plate supplemented with sucrose (20 g/l) and ampicillin (50 mg/ml) and cultured at 37°C for 2 days. Spraying GOD-PAP (Böeringer Mannheim) onto the plate allowed the selection of 13 colonies which formed red halos around themselves. Further selection was on the colonies which could be of sucrase activity as well as of levan formativity as measured according to the instruction of Gay et al.

(Gay et al., J. Bacteriol. 153, 1432(1983)). From the *E. coli* colonies, plasmids were isolated and named "pZL8". *E. coli* DH5 α bearing the plasmid was deposited in Korean Collection for Type Cultures, Korean Research Institute of Bioscience and Biotechnology on Dec 14, 1993 at deposition No. KCTC 8546P.

5

Gene Identification by Levan Formation

Cultivation of the *E. coli* bearing pZL8 at 37°C for 3 days in an LBS broth (LB broth supplemented with sucrose at 5%) resulted in the detection of polymers similar to those of levan. The culture was centrifuged and the supernatant was added with three volumes of cold ethanol to precipitate polymers. They were dissolved in distilled water and precipitated with ethanol. This ethanol precipitation procedure was carried out twice more. The raw polymers thus obtained were separated by thin layer chromatography (TLC) on silica gel developing with n-butanol/pyridine/water (8/1/1), followed by the coloration with a 1% anisaldehyde-sulfuric acid solution.

Because levan, a macro-molecule with a molecular weight of 5×10^7 Daltons, cannot move on TLC, it was hydrolyzed according to the instruction of Tanaka (Tanaka et al., J. Biochem. 90, 521 (1981)). 100 μ l of the raw polymer solution was boiled for 15 min, along with 50 μ l of 2.5 % oxalic acid, and the hydrolyzed solution was subjected to TLC. The acid hydrolysate was extended for 15 min, only fructose was observed. There were difficulties in analyzing the sugar components with accuracy by TLC because the R_f value was 0.46 for glucose, 0.52 for fructose, and 0.4 for sucrose. To compensate for this, HPLC analysis was executed.

20 μ l of each of the hydrolysate samples was filtered through a 0.2 μ m membrane and subjected to HPLC with the aid of an HPLC apparatus, such as sold by Waters, identified as "Model R401", by flowing water at a rate of 0.6

ml/min through a column carbohydrate HPX-87C (Bio-Rad). Detection was conducted using RI (Oven Temp. 85-90°C). All of the samples showed two peaks: a peak was read at 4.19 min for oxalic acid and the other peak at 9.29 min for sucrose. These data, therefore, demonstrated that the polymer obtained
5 above was composed of fructose residues. In addition, no inulin ($\beta(2-1)$ linked polyfructan) formative activity was observed in *Z. mobilis* (Toran et al., *J. Biotechnol. Lett.* 7, 527(1985)). Consequently, it was made certain that the polymers formed in the *E. coli* bacteria bearing the plasmid pZL8 were levan ($\beta(2-6)$ linked polyfructan) (Viikari, L., *CRC Critical Reviews in Biotechnology*
10 7, 237 (1988)) and a levansucrase gene was introduced in the plasmid pZL8.

Gene Identification by Gene Mapping

The recombinant plasmid obtained above was digested with various restriction enzymes and electrophoresed on 1% agarose gel. Based on the
15 DNA fragment data on the agarose gel, a restriction enzyme map of the plasmid pZL8 was drawn, providing the information that the genomic DNA introduced in the plasmid pZL8 was 4.5 kb long.

To be used as a probe, the plasmid pZL8 was labeled with a DNA labeling kit (Böeringer Mannheim). Separately, the *Zymomonas mobilis*
20 genomic DNA prepared above was cut with Restriction enzymes, *EcoRI*, *EcoRV*, *HindIII* and *NcoI* and electrophoresed on 1% agarose gel. Then, the genomic DNA fragments on the agarose gel were transferred to a nylon membrane and hybridized with the probe in accordance with the teaching of Southern (Southern, *J. Mol. Biol.*, 98, 503 (1975)). Referring to the instruction enclosed in the
25 DNA labeling kit, coloration was achieved. Because the plasmid pZL8 has a unique restriction enzyme site for each of *EcoRV*, *HindIII* and *NcoI*, the DNA sequence of 4.5 kb introduced into the plasmid pZL8 must be from *Z. mobilis*.

Z. mobilis ZM1 was cultured at 30°C for 24 hours in a YPS medium and the multiplied cells were harvested, suspended in a 20 mM phosphate buffer (pH 6.8), shaken at 30°C for 15 min, and centrifuged according to the introduction of Yanase (Yanase *et al.*, *Biosci. Biotech. Biochem.* 56, 1335 (1992)). The

supernatant was used as a crude enzyme solution.

In this crude solution, ammonium sulfate was saturated up to 50 %, to precipitate proteins which were recovered by centrifugation at 8,000xg for 20 min. The protein mass was dissolved in a 0.02 M phosphate buffer (pH 6.8), followed by dialysis in the same buffer. In this regard, elution was conducted at a rate of 0.5 ml/min through a column (2.5x10 cm) charged with a weak anion exchange resin (DEAE-Toyopearl 650M). In a linear concentration gradient of NaCl from 0 to 0.5 M, the eluate at 0.3 M was collected. The eluate was concentrated and purified followed by Hydroxyapatite column chromatography. After being concentrated, The protein was allowed to precipitate with 20% saturated ammonium sulfate. And finally the concentrate was loaded on a gel filtration column (Superose 12, Pharmacia) to elute a fraction containing a molecular weight of 91,000. The final purification yield was 18.3 fold of the crude enzyme from *Z. mobilis*, with 16.5% of the enzyme recovered in the preparation step (Table 1). The solution was used as a levansucrase solution.

TABLE 2. roductionP Amounts of Levan According to pH of Buffer (g/l)

TABLE 1. Summary of levansucrase purification steps from *Z. mobilis*

Step	Volume (ml)	U total	Protein (mg/ml)	Spec. Act. (U/mg)	Yield (%)	Purifi. Fold
Cell washed	1,300	-a	0.35	-	-	-
1st (NH ₄) ₂ SO ₄	115	-	1.28	-	-	-
Ion-exchange	38	4.35	0.57	0.21	100	1.00
Hydroxyapatite	20	2.58	0.41	0.31	65	1.52
2nd (NH ₄) ₂ SO ₄	2	0.96	0.46	1.04	21	5.07
Superose 12	1.5	0.72	0.13	3.75	16.5	18.3

20 a: could not be determined.

With the aid of a protein-peptide sequencing system (Applied Biosystems, Model 477A), the amino acid sequence of the purified levansucrase was determined at its N-terminal in the Edman degradation procedure. As a result, a stretch of seven amino acid residues, Met-Leu-Asn-Lys-Ala-Gly-Ile, was sequenced, reflecting the corresponding base sequence of the DNA. In particular, the *levU* gene was revealed to have no base sequences which correspond to the signal peptides, which are usually found in secretory proteins. The nucleotide and amino acid sequences of the levansucrase gene from *Z. mobilis* was registered in the GenBank, U.S.A. (Accession No. AF081588).

10

EXPERIMENTAL EXAMPLE 2

Production of Levansucrase

15 Step 1 Construction of Expression Vector

E. coli KCTC 8546P was cultured at 37°C overnight in an LB medium (yeast extract 0.5 %, trypton 1 %, NaCl 1 %), after which plasmid pZL8 was extracted from the culture, according to the method of Maniatis *et al.* (Maniatis *et al.*, *Molecular Cloning: A Laboratory manual*, 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., U.S.A.).

A levansucrase gene 1296 bp long was amplified by a PCR which employed the following primers 1 and 2 with the plasmid pZL 8 serving as a template:

25 Primer 1: 5'-CGC CGG ATC CAC ATG TTG AAT AAA GCA GGC-3'

Primer 2: 5'-CGC GGA TCC ACA TGT GCA TAA TCA GAA ACG TC-3'

A reaction mixture comprising a 10x Taq polymerase buffer (10 ul), a 10x dNTP mix (10 ul) (dATP, dCTP, dGTP, and dTTP, 2 mM each), the primers 1 and 2 (1 ul (100 pM), each), the plasmid pZL8 DNA (5 ul (20 ng)), and deionized water (72 ul) was thermally treated, along with a mineral oil (100 ul), at 95°C for 5 min to denature the DNA. The reaction mixture was added with a Taq polymerase (1 ul (5 U)) after being cooled to 72°C and then, allowed to undergo 25 thermal cycles in which heating processes were performed in the order of at 95 °C for 1 min, at 55°C for 2 min and at 72°C for 3 min.

The PCR product thus obtained was digested with the restriction enzyme *Afl*III and introduced into the *Nco*I site of the plasmid pET3d (Stratagene) to construct the *E. coli*-expression plasmid pEL11.

Step 2 Transformation of *E. coli*

E. coli DH5 α and BL21 (DE3) were separately cultured at 18°C for 36 hours in 30 ml of an SOB medium (Trypton 2%, Yeast extract 0.5%, NaCl 10 mM, calcium chloride 2.5 mM, magnesium chloride 10 mM, magnesium sulfate 10 mM), followed by centrifugation to harvest the bacteria. Subsequently, the cultured bacteria were separately suspended in 10 ml of a TB buffer (Pipes 10 mM, manganese chloride 55 mM, calcium chloride 15 mM, potassium chloride 250 mM), followed by centrifugation to collect the cells. The cell mass was resuspended in 12 ml of a TB buffer and after being added with DMSO (dimethyl sulfoxide) at a concentration of 7 %, the suspension was kept on ice and stored at an aliquot of 400 ul in a liquid nitrogen tank.

After being thawed, the *E. coli* DH5 α was added with 10 ul of the plasmid pEL11 constructed in the Step 1 and kept on ice for 30 min, followed by heat shock at 42°C for 30 sec for transformation. The transformed cell mixture was added with 800 ul of an SOC medium, vigorously agitated at 37°C for 60

To the cell pellet which was obtained by centrifuging the culture, a 100 mM Tris buffer (pH 7.0) was added at an amount of 1/10 volume of the culture, and the suspended cells were homogenated by sonicating three times for 30 sec with the aid of a sonifier (Branson), followed by centrifugation at 12000xg for 60 min. The supernatant thus obtained was added with ammonium sulfate to the concentration of 20 % and centrifuged to give above 94 %-pure levansucrase. This crude enzyme mass was found to have a levansucrase activity of 7.8 U/ml, if harvested at 4 hours after the IPTG induction, as measured according to the instruction described in Song et al. (song and Rhee, *Biotechnol. Lett.* 16, 1305 (1994)) Example 2. It amounted to 30 % of the total quantity of the proteins produced from the *E. coli*.

A PCR was carried out using Primer 1 and Primer 3 (5'-CGA CAT GTT AGT GAT GGT GAT GGT GAT GTA AAG ACA GGG CTG-3') while the plasmid pZL8 isolated from *E. coli* KCTC 8546P served as a template. Using the PCR product, the same procedure as Experimental Example 2 was repeated to construct the plasmid pEL12 capable of expressing the levansucrase carrying histidine residues at its C-terminus in *E. coli*, which was then used for the transformation of *E. coli* BL21 (DE3).

The transformed *E. coli* BL21 (DE3)/pEL12 was deposited in Korean Collection for Type Cultures, Korean Research Institute of Bioscience and Biotechnology on April 25, 1995 at deposition No. KCTC 8861P.

EXAMPLE 3

Ni-NTA resin (Quagen, U.S.A.) was charged in a 1.5x20 cm column through which a 50 mM phosphate buffer (pH 8.0) was then flowed to give a column for metal ion-affinitive chromatography.

E. coli KCTC 8861P of Example 2 was cultured in the same manner as the Step 3 of Example 1 and sonicated. The cell homogenate was loaded on the column which was then washed with a buffer (50mM sodium phosphate, 300 mM NaCl, 10% glycerol, pH 6.0), followed by detaching the enzyme from the resin by flowing a 0.3 N imidazole solution (pH 7.0). The result is given in Fig. 4. As shown in Fig. 4, the enzyme was purified with above 95% of homogeneity (lane 2), from the total protein of *E. coli* lysate(lane 1).

EXAMPLE 4

Using the levansucrase carrying histidine residues at its C-terminal,

In 10 ml of a 50 mM acetic acid buffer (pH 5.0) was dissolved sucrose to the concentration of 10 % and was added 1.05 U of the crude enzyme obtained in Experimental Example 2. The resulting mixture was allowed to react at –

3°C, 0°C, 5°C, and 10°C for up to 200 hours, after which a measurement was made of the amounts of the levan produced according to the condition parameters.

The results are given in Table 3, below. As apparent from the data, a reaction temperature of 10°C was the best condition for the crude enzyme to produce levan until 50 hours of the reaction.

TABLE 3. Production Amounts of Levan According to Reaction Temp. (g/l)

Temp.	Time period					
	4	10	27	46	78	167
-3 °C	7.3	10.5	20.5	30.0	34.5	30.5
0 °C	10.1	15.7	29.5	36.5	37.2	32.3
5 °C	11.3	22.2	33.2	36.4	38.4	33.0
10 °C	15.2	29.3	34.9	36.7	37.5	29.7

10

EXAMPLE 6

In 10 ml of a 50 mM acetic acid buffer (pH 5.0) was dissolved sucrose to the concentrations of 5-40 % and was added 2.08 U of the crude enzyme prepared in Experimental Example 2. The resulting mixture was allowed to react at 10°C for up to 60 hours, after which a measurement was made of the amounts of the levan produced according to the condition parameters.

The results are given in Table 4, below. As apparent from the data, the levan amounts produced in a sucrose concentration of 30 % were greater than in any other sucrose concentration.

TABLE 4. Production Amounts of Levan According to Sucrose Concentration (g/l)

Sucrose Concentration	Time period					
	2	11	18	25	36	59
5	12.1	19.0	21.8	21.6	21.4	20.8
10	14.8	38.2	36.7	39.1	34.2	34.9
20	14.1	51.4	56.7	52.9	51.4	52.0
30	14.6	52.4	71.1	66.9	69.8	59.4
40	11.5	39.1	59.8	58.8	60.0	56.4

5

EXAMPLE 7

In 5 ml of a 50 mM acetic acid buffer (pH 5.0) was dissolved sucrose to the concentration of 10 % and were added 0.42U, 1.05 U, and 2.08 U of the crude enzyme prepared in Experimental Example 2. The resulting mixtures were allowed to react at 10°C for up to 150 hours, after which a measurement was made of the amounts of the levan produced according to the condition parameters.

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The results are given in Table 5, below. It is apparent from the data that the levan is produced at larger amounts by the greater activity of the enzyme.

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TABLE 5. Production Amounts of Levan According to Enzyme Concentration (g/l)

Enzyme Concentration	Time period				
	4	12	48	96	144
0.42U	2.5	4.0	21.7	35.7	39.0
10.5U	4.9	12.9	34.9	48.5	42.2
20.8U	24.9	44.9	49.0	49.2	46.2

EXAMPLE 8

In 5 ml of a 50 mM acetic acid buffer (pH 5.0) was dissolved sucrose to the concentration of 20 % and were added 2.08 U of the crude enzyme prepared in Experimental Example 2. The resulting mixtures were allowed to react at 10°C for up to 36 hours, after which a measurement was made of the amounts of the levan and other saccharides produced according to the condition parameter.

The results are given in Table 6, below. It is apparent from the data that the levan is produced at larger amounts by the greater activity of the enzyme.

TABLE 6. Production Amounts of Levan According to Reaction Time (g/l)

Saccharides	Time period					
	0	6	11	18	25	36
Levan	0	28.3	51.4	56.7	52.9	51.4
Residual Sucrose	200	161	125	120	98	10
Glucose	0	237	36.0	50.4	74.6	104.8
Fructose	0	2.5	5.8	6.2	6.5	7.2

EXAMPLE 9

15

In 10 ml of a 50 mM acetic acid buffer (pH 5.0) was dissolved raw sugar (DaeHan Sugar Co.) to the concentration of 20 % and were added 1.05 U of the crude enzyme prepared in Experimental Example 2. The resulting mixtures were allowed to react at 10°C for up to about 91 hours, after which a measurement was made of the amounts of the levan and other saccharides produced. Levan was produced at an amount of 12.5 g/l after 11 hours of the reaction, 25.0 g/l after 25 hours, and 45.8 g/l after 91 hours.

20

EXPERIMENTAL EXAMPLE 4

Selection of Strains Containing Levan Fructotransferase Capable of Producing

5 DFA IV from Levan

Strains were selected from samples taken from soil in Korea. 0.5 grams of each of the soil samples was suspended in 9.5 ml of distilled water and the suspensions were successively diluted to 10-fold, 100-fold and 1000-fold. 200 ul of each of the dilutions was spread on the surface of a selective agar plate, and incubated at 30°C for 72 hours in an incubator. The selective agar medium contained 0.5 % (w/v) of levan as a major carbon source, along with 0.3 % of NaNO₃, 0.05 % of MgSO₄, 0.02 % of MnCl₂, 0.1 % of K₂HPO₄ and 1.5 agar/L. A solution of a predetermined amount of levan in water was passed through a 0.45 micron filter and the filtrate was mixed with the other nutrients before being added to a sterilized medium. After the incubation, colonies around which halos had been formed were primarily selected as being capable of decomposing levan.

Inocula picked from the primarily selected colonies were inoculated in selective broths, each containing levan as a major carbon source, and cultured at 30°C for 72 hours in an incubator. Investigation was made into the growth of the strains inoculated and the metabolites resulting from the metabolism of levan through thin layer chromatography (TLC). Of the 250 strains primarily selected, 8 strains of microbes were found to produce difructose dianhydride. Finally, one strain which stably produced difructose from levan with high enzyme activity was selected and named "K2032".

Identification of Strain K2032

The identification of the selected strain K2032 was achieved by examining its biochemical characteristics and the composition and content of its fatty acids and quinones and comparing them with the database previously accumulated. Many experiments revealed that strain K2032 is Gram-variable and is able to utilize glucose, fructose, galactose, arabinose, mannitol, xylose, ribose, sorbitol, cellobiose, glycogen, sucrose, acetic acid, propionic acid, and salicin, but not rhamnose, malonate, adipic acid, N-acetyl glutamic acid, and adipic acid. As for its intracellular fatty acid composition, the bacteria comprises C15 anteiso/C17 anteiso/C16 iso at a ratio of 51%/14.5%/3.6% with C16/C14 being at a ratio of 17.8%/7.1%. As a result of the analysis of quinones, which are important components for the chemotaxamomy of microbes, the bacteria contained menaquinone MK-9(H₂).

With the analysis results, the strain K2032 was identified as *Arthrobacter ureafaciens*, and thus, named *A. ureafaciens* K2032 (Table 7).

TABLE 7. Phenotypic and chemotaxonomic characteristics of strain K2032

	Gram reaction	variable (+ when young)
20	Acid from glucose	-
	Nitrate reduction	-
	Starch hydrolysis	-
	Growth in 10% NaCl	-
	Utilization of D-glucose, D-fructose, D-galactose,	+
25	L-arabinose, D-mannitol, D-xylose, D-ribose	
	D-sorbitol, cellobiose, glycogen, sucrose,	
	acetic acid, propionic acid, salicin	
	Utilization of L-rhamnose, malonate, adipic acid,	-
	N-acetyl glutamic acid, L-serine	
30	Cellular fatty acid;	
	C15 anteiso/C17 anteiso/C16 iso	51%/ 14.5%/ 3.6%
	C16/C14	17.8%/ 7.1%
	Major menaquinone	MK-9(H ₂)

EXPERIMENTAL EXAMPLE 5

Purification of Levan Fructotransferase from *Arthrobacter*

5 *A. ureafaciens* K2032 was inoculated in a selective broth containing levan as
a major carbon source and cultured at 30°C for 72 hours in an incubator.
Investigation into the growth and levan fructotransferase activity of the
bacteria provided the knowledge that the largest enzyme activity is obtained
after 10 hours of the incubation. From the culture incubated for 10 hours,
10 the enzyme was purified (Fig. 5). In this regard, the purification was
achieved by successively using acetone precipitation, ion exchange
chromatography (DEAE 650-M and Mono Q), and gel filtration
chromatography, resulting in producing levan fructotransferase with a
specific activity of 2269 U/mg protein at an amount of 1.1 mg at a yield of
15 29.8 % (Table 8).

TABLE 8. Summary of levan fructotransferase purification from *A. ureafaciens* K2032.

Purification step	Volume (ml)	Total activity (U)	Protein (mg)	Spec. Act. (U mg ⁻¹ protein)	Purification Fold	Yield (%)
Culture supernatant	3,800	8,360	182.3	45.9	1.0	100
Acetone precipitation	700	5,670	71.3	79.5	1.7	67.8
Ion-exchange adsorption	5.5	4,821	5.0	964.2	21.1	57.7
2 nd Ion-exchange adsorption	3.2	3,416	2.6	1313.8	29.2	40.9
Gel permeation (Superose 12)	1.2	2,496	1.1	2269.0	49.8	29.8

Characterization of the Purified Enzyme

The purified enzyme was 51,000 in molecular weight as measured by SDS-PAGE while being measured to have a molecular weight of 96,000 by gel filtration chromatography. Thus, it was identified as being a dimer in aqueous conditions. An amino acid analysis showed that the enzyme had at its C-terminus a stretch of the amino acid sequence SAPGSLRAVYHMTTPPSGXLXDOQ wherein X residues are not identified. Maintaining its stability in a pH range of 4-10.5, the enzyme showed optimal activity at around pH 6.0. Also, it was of optimal activity at 55 °C. After being allowed to stand at 50°C for 30 min, the enzyme was observed to have a remnant activity of 90 % or greater (Table 9). Whereas being inhibited by Mn^{2+} , Fe^{2+} and Hg^{2+} , the activity of the enzyme was enhanced by Na^{2+} and Ca^{2+} .

TABLE 9. Effect of additives on the activity of levan fructotransferase from *A. ureafaciens* K2032

Additive	Concentration (mM)	Relative activity (%)
Control	-	100
NaCl	1, 10	125, 142
NH ₄ Cl	1	46
MnCl ₂	1	9
CaCl ₂	1, 10	156, 181
NiCl	1	74
ZnCl ₂	1	23
LiCl	1	92
MgCl ₂	1	63
FeCl ₂	1	4
HgCl ₂	1	0
KCl	1	89
EDTA	1, 10	95, 94
SDS	1, 10	101, 32

EXPERIMENTAL EXAMPLE 6

Isolation of Genomic DNA from Arthrobacter

Isolation of a gene coding for a levan fructotransferase from *A. ureafaciens* was performed using a genome DNA isolation kit, such as that sold by Bio 101, identified as "G NOME™ DNA ISOLATION KIT", according to its instruction. First, *A. ureafaciens* K2032 was inoculated in 50 ml of a selective broth and cultured for 24 hours, followed by centrifugation at 3,000 rpm for 5 min to harvest cells. To the cell pellet was added the cell suspension solution (10 mM Tris-HCl (pH 8.0), 0.1 M EDTA (pH 8.0)) to the volume of 1.85 ml. This cell suspension was well mixed with 100 ul of the cell lysis/denaturing solution (0.5% SDS), along with 50 ul of the RNase solution, and incubated at 55 °C for 15 min. To remove proteins from the sample, 25 ul of the protease was added and incubated at 55°C for 1 hour. After 500 ul of the salt out solution was added, the mixture was aliquoted in 1.5 ml tubes which were then cooled at 4°C for 10 min. Following centrifugation at 12000 rpm for 10 min, the supernatant was transferred to a 15 ml tube, in which 2 ml of TE buffer and 8 ml of 100 % ethanol were added to induce DNA precipitation. The DNA precipitates were collected by centrifugation at 12,000 rpm for 15 min, dried in the air and dissolved in a TE buffer.

Cloning of Levan Fructotransferase-Coding Gene

The genome DNA prepared from *A. ureafaciens* was digested with various restriction enzymes and electrophoresed on 1% agarose gel, after which DNA fragments in a size range of 3-4 kb were eluted from the agarose gel. The partial genomic DNA fragments were ligated into the cloning vector pBluescript KSII⁺ which was previously cut with *Bam*HI. This recombinant

plasmid was transformed into *E. coli* DH5 α . Of the transformants, the cells carrying a gene coding for a levan fructotransferase were selected as follows.

First, the -V-Y-H-M-T-P- in the N-terminal amino acid sequence of the K2032 strain and the amino acid sequence (-E-C-P-D-L-F-, p2 primer), which
5 was found to be in a homology relationship with levan fructotransferase, were used to synthesize degenerated primers (p1: 5'-GTNTAYCAYATGACNCC and p2: 5'-AABAYYTCSGGYCARTC). Using these primers, a standard PCR was carried out with the genomic DNA of K2032 bacteria serving as a template. The PCR products ranging, in size, from 600 to 650 bp were eluted from the gel
10 and introduced into shuttle vectors for *E. coli*. As a result of restriction enzyme gene mapping, the DNA fragments introduced were identified as being divided into four kinds and the vectors carrying these DNA fragments were called pDA11, 17, 18 and c8, respectively. In addition, base sequencing analysis of the four DNA fragments showed that the DNA fragment inserted in pDA11 and
15 the levan fructotransferase gene of *A. nicotinovorans* are in high homology (85 %) relationship. Therefore, the DNA fragment introduced in pDA18 could be used as a probe for the Southern hybridization with the DNA prepared from *A. ureafaciens* K2032 with the aim of detecting the levan fructotransferase gene anchored in the bacteria. Before the Southern hybridization, the genomic DNA
20 of *A. ureafaciens* K2032 was digested with various restriction enzymes. As a result of the Southern hybridization, a signal was detected in a 5.6 kb DNA fragment upon digestion with *Cla*I, in a 8.0 kb DNA fragment upon digestion with *Pst*I, and a DNA fragment longer than 10 kb upon digestion with *Bam*HI. Accordingly, the genomic DNA was cut with either *Cla*I or *Pst*I and
25 electrophoresed on a 1% agarose gel, after which DNA fragments in a size range of 5-10 kb were eluted from the agarose gel. The partial genomic DNA fragments were ligated into the cloning vector pBluescript KSII⁺ which was

previously cut with *Cla*I or *Pst*I. These recombinant plasmids were transformed into *E. coli* DH5 α . Of the transformants, the cells carrying a gene coding for a levan fructotransferase were selected by Southern hybridization. Finally, when cutting with *Cla*I, there was obtained plasmid pDCla carrying a
5 5.6 kb DNA fragment. On the other hand, when cutting with *Pst*I, there was obtained plasmid pDpst carrying a 8.0 kb DNA fragment.

Isolation, Analysis and Subcloning of the Recombinant Plasmids

DNA preparation from the transformed *E. coli* was carried out by an
10 ordinary boiling method and with the aid of a commercially available kit. To analyze the recombinant plasmids, they were digested with restriction enzymes and electrophoresed on 1% agarose gel.

The restriction enzyme gene mapping of the DNA fragments introduced in pDCla and pDpst, the analysis of gene loci, and the Southern blotting with
15 DNA fragments showed that pDCla and pDpst carried an N-terminal half and a C-terminal half of the gene of interest with an overlapping stretch of about 200 bp therebetween. No enzymatic activity was detected from the bacteria which anchored the two plasmids respectively. According to a base sequencing analysis, the sites for restriction enzymes *Pst*I, *Apa*I and *Not*I were found to be
20 present in both of the plasmids. By taking advantage of these common restriction enzyme sites, there was constructed plasmid pDF8 which has a complete open reading frame (ORF). A DNA fragment 3.5 kb long was introduced in the plasmid pDF8 and enzymatic activity was detected from the bacteria anchoring this plasmid.

25

Base Sequence of the Cloned Levan Fructotransferase Gene and Its Putative Amino Acid Sequence

In order to analyze the nucleic acid base sequence of the 3.5 kb DNA introduced into pDF8, DNA ladder fragments which showed a regular distance of about 300-400 bp were made and subjected to a sequencing process. As a result, a sequence of 3345 bases was ascertained and found to have only one ORF. Using the BLAST search, which is available from the Internet, the ORF was investigated for homology. In result, a levan fructotransferase gene was detected as being high in the homology with the ORF, and thus, it was named *lftA*. Fig. 6 shows the base sequence of *lftA* and the amino acid sequence deduced therefrom.

The newly isolated levan fructotransferase structural gene is composed of 1566 bp which corresponds to 521 aa. The gene of the present invention has a signal sequence consisting of 99 bp (corresponding to 33 aa) at its N-terminus and therefore, the putative completed protein may be about 56 kDa in molecular weight. Its isoelectric point was found to be pH 5.15. This levan fructotransferase gene is longer by 15 bases (5 amino acids) than is the levan fructotransferase gene derived from *A. nicotinovorans* GS-9. These two genes are 81% in the homology of amino acid sequence (76% in the homology of base sequence). Consequently, the levan fructotransferase gene of *A. ureafaciens* K2032 was identified as being new and its base sequence was registered in the GenBank, U.S.A. (Accession No. AF181254).

EXPERIMENTAL EXAMPLE 7

Development of Highly Expressable, Transformed E. coli Carrying Recombinant Levan Fructotransferase Gene

Using the restriction enzyme *Pst*I, the *lftA* gene was cut at its signal sequence, followed by Klenow treatment to give a blunt end. The *lftA* gene

was divided by cutting with *NotI*. The Klenow-treated DNA fragment was further digested with *SmaI* and ligated to the pUC118 vector which was previously treated with CIP, so as to construct pUDFA18. This was introduced into *E. coli* DH5 α . Fig. 7 shows the plasmid pUDFA18 which is obtained by inserting the *lftA* gene in the expression vector pUC118. Fig. 8 shows the total protein pattern of *E. coli* JUD81 in SDS-PAGE. The *E. coli* JUD81 is the *E. coli* DH5 transformed with pUDFA18.

EXPERIMENTAL EXAMPLE 8

10

Production of DFA IV from Levan by Use of Recombinant Levan Fructotransferase

In 1,000 ml of warm water was dissolved 20 g of levan, cooled and controlled to pH 6.5 with a phosphate buffer. To the resulting solution, *E. coli* JUD81 homogenate was added and allowed to react at 37°C. Fig. 9 shows the change of the amounts of DFA IV and other saccharides in the reaction mixture in accordance with reaction time period. After 40 hours of the reaction, the reaction mixture was allowed to stand in hot water for 5 min to inactivate the remaining enzyme and loaded onto a charcoal column (diameter 6.5 cm, height 20 cm). The column was washed with distilled water until no saccharides were detected in the washings. The column was again washed with 1,000 ml of 5 % ethanol and then with 1,000 ml of 25 % ethanol to elute the absorbed DFA IV. The effluents containing DFA IV were collected and concentrated to a volume of 30 ml by use of a rotary evaporator. To the concentrate, 100 % ethanol was added to the final ethanol concentration of 95 % or higher, so as to crystallize DFA IV. The DFA IV precipitates were washed many times with pure ethanol

The present invention has been described in an illustrative manner, and it is to be understood that the terminology used is intended to be in the nature of description rather than of limitation. Many modifications and variations of the present invention are possible in light of the above teachings. Therefore, it is to be understood that within the scope of the appended claims, the invention may be practiced otherwise than as specifically described.

CLAIMS:

1. A novel microorganism *A. ureafaciens* K2032, which shows an activity of selectively producing difructose dianhydride IV from levan and an activity of degrading levan.

5

2. A novel levan fructotransferase of the following amino acid sequence 1, derived from *A. ureafaciens* K2032, which can hydrolyze levan to selectively produce difructose dianhydride IV:

10 1 M T P A I S R R A V L Q G A G A G A L A L I F G G A V P P A
 31 A R A S A P G S L R A V Y H M T P P S G W L C D P Q R P V T
 61 T H G A Y Q L Y Y L H S D Q N N G P G G W D H A S T T D G V
 91 A F T H H G T V M P L R P D F P V W S G S A V V G T A N T A
 121 G F G A G A V V A L A T Q P T D G V R K Y Q E Q Y L Y W S T
 151 D G G F T F T A L P D P V I V N T D G R A A T T P A E I E N
 181 A E W F R D P K I H W D T A R G E W V C V I G R L R Y A A F
 211 Y T S P N L R D W T L R R N F D Y P N H A L G G I E C P D L
 241 F E I T A D D G T R H W V L A A S M D A Y G I G L P M T Y A
 271 Y W T G T W D G E Q F H A D D L T P Q W L D W G W D W Y A A
 301 V T W P S I D A P E T K R L A I A W M N N W K Y A A R D V P
 331 T D A S D G Y N G Q N S I V R E L R L A R Q P G G W Y T L L
 361 S T P V A A L T N Y V T A T T T L P D R T V D G S A V L P W
 391 N G R A Y E I E L D I A W D T A T N V G I S V G R S P D G T
 421 R H T N I G K Y G A D L Y V D R G P S D L A G Y S L A P Y S
 451 R A A A P I D P G A R S V H L R I L V D T Q S V E V F V N A
 481 G H T V L S Q Q V H F A E G D T G I S L Y T D G G P A H F T
 511 G I V V R E I G Q A I *

3. A novel levan fructotransferase gene, which has a base sequence
 30 coding for the amino acid sequence of claim 2.

4. A novel levan fructotransferase gene as set forth in claim 3, wherein the base sequence is the following sequence 2:

1 GCGGTGCACCCGACTTCCCTCGACGACCAACCGTCCCTACCGCCGACCGCCCGGCCGACTGCTCCTCAGCCTAGACGGGCCCTC
5 91 CTCGAGGTCTTCGTGGGGACGGTGAGGCGACTGCGTCGAACCTGGTCTCTCTGGGGGCCGGCGGTGTACCGCGAGCCTCGAGACGGCA
181 CGGCCAGGAACCGTGACGTGACCGGATCGACGTGAGGGGCCAGCGATGCTGACGCCCTGAACCTGCCGCCGTTCTGGGTGACGA
271 GCGTCCCACCCCGACAGCTCTCTTCTACCGCTGCCGAACAGGGTGGACGCTTCGTGCGCCACCCGTCACGAGAGGAACAGCA
361 ATGACGCGCGCATCTCACGCCGCGCTGCTCCAGGGAGCCGGCGCGGAGCACTCGCCCTGATCTTCGGCGGTGCTGTGCGCGCTGCA
451 GCCCGGGCATCCGCTCCGGGCTCGCTCCGTGCCGTCTACACATGACGCCCCAGCGGCTGGCTCTGCGACCCCCAACGCCCGGTACC
10 541 ACCCAGCGGCTACAGCTGTACTACCTGCACTCCGACCAAGCAACCGGCCCGGGCGGTGGGACCACGCGAGCAGACCGACGGCGTC
631 GCCTTCACGACACCGGACCGTGTATGCCGCTCGGCGCCGACTTCCCCGTGTGGTCCGGGTGGCGGTGCTGCGCACCCGCAACACGGCA
721 GGGTTCGGCGCGCGCGCGGTGCTGCGCTCGCGACCCAGCGGACCGGCGTCCGCAAGTACCAGGAGCAGTACCTCTACTGGTCGACC
811 GACGGCGGGTTCACGTTACCGCCCTGCCGACCCGTCATCGTCAACACCGACGGTTCGCGCGCCACCAACGCGCGGAGATCGAGAAC
901 GCCGAGTGGTTCGCGACCCCAAGATCCACTGGGACACCGCCCGCGGAGAATGGGTCTGCGTCATCGGACGACTGCGGTACGCCGCGTTC
15 991 TACACCTCGCGCAACCTGCGGACTGGACACTTCGCGCAACTTCGACTACCCGAACACCGCCCTCGGCGGCATCGAGTGCCCCGACCTG
1081 TTCGAGATACCGCAGACGACGGGACACGCCACTGGGTGCTCGCGCCAGCATGGACGCCCTACGGCATCGGCCTCCCCATGACGTACGCC
1171 TACTGGACAGGCACCTGGGACGGCGAGCAGTTCACGCGCGACGACCTCACCCGCAATGGCTCGACTGGGGCTGGGACTGGTACCGCGGC
1261 GTCACCTGGCCATCGATCGACGCGCCGAGACCAAGCGCTCGCCATCGCGTGGATGAACAAGTACGCGCACGCGACGTCCCC
1351 ACCGACGCATCCGACGGCTACAACGGGAGAACTCGATCGTCCGCGAGCTGCGGCTCGCCCGACAGCCTGGCGGTGGTACACCTCCTG
20 1441 AGCACCCCGTGGCAGCGCTGACGAACACTGACCGCCACCACTACCTCCCGACCGGACCGTGCAGCGCAGCGCGTCTGCCATGG
1531 AACGGACGCGCATAAGAGATCGAGCTCGACATCGCCTGGGACACCGCGACGAACGTCGGCATCTCGGTGGGCCGCTCCCCGACGGAACC
1621 CGGCACACGAACATCGGCAAGTACGGAGCAGACCTGTACGTGACCGGAGGACCTCCGACCTCGCCGGTACTCGCTCGCCCTACTCG
1711 CGAGCGCGCGCCCATCGACCCCGCGCCGATCCGTGCACCTGCGCATCTCTGTCGACACCCAGAGCGTCGAGTCTTCGTCAACGCC
1801 GGCCACACCGTGTCTCCAGCAGGTCCACTTCGCGGAGGGGACACGGGAATCTCGCTCTACACCGACGGCGGCCCGCACACTTACC
25 1891 GGCATCGTCTCGCGAGATTGGCCAGGCGATAGGCGATGCACACCACACCGCTACCGAAGCGCGCCCGGGAGACGACGGCGGAC
1981 AATCGACACGTCCTCGTCGT

5. A novel levan fructotransferase gene as set forth in claim 3, wherein the base sequence is the following sequence 3:

30 1 GCGGTGCACCCGACTTCCCTCGACGACCAACCGTCCCTACCGCCGACCGCCCGGCCGACTGCTCCTCAGCCTAGACGGGCCCTC
AvaI AvaI
91 CTCGAGGTCTTCGTGGGGACGGTGAGGCGACTGCGTCGAACCTGGTCTCTCTGGGGGCCGGCGGTGTACCGCGAGCCTCGAGACGGCA
181 CGGCCAGGAACCGTGACGTGACCGGATCGACGTGAGGGGCCAGCGATGCTGACGCCCTGAACCTGCCGCCGTTCTGGGTGACGA

271 GCGCTCCACCCCGACAGCTCTCCTTCTACCGCTGCCGAACAGGGTGGACGCTTCGTGCGGCCACCCGTCCACGAGAGGAACACGCA

PstI

361 ATGACGCCGGCCATCTCACGCCGCGCGTGTCCAGGGAGCCGGCGCGGAGCACTCGCCCTGATCTTCGGCGGTGCTGTGCCGCTGCA

I M T P A I S R R A V L Q G A G A G A L A L I F G G A V P P A

5 451 GCCCGGGCATCCGCTCCGGGCTCGCTCCGTGCCGTCTACACATGACGCCCCCAGCGGCTGGCTCTGCGACCCCAACGCCCGGTCAAC

31 A R A S A P G S L R A V Y H M T P P S G W L C D P Q R P V T

541 ACCCAGCGCGCTACAGCTGTACTACGTGCACTCCGACCAGAACAACGGCCCCGGCGGCTGGGACCACGGAGCACGACCGACGGCGTG

61 T H G A Y Q L Y Y L H S D Q N N G P G G W D H A S T T D G V

631 GCCTTCACGACACCGGCACCGTGTATGCCGCTGCGGCCGCACTTCCCGTGTGGTCCGGGTGCGCGGTGCTGCGCACCGGAACACGGCA

10 91 A F T H H G T V M P L R P D F P V W S G S A V V G T A N T A

721 GGGTTCGGCGCGCGCGGTGCTGCGGCTCGCGACCCAGCCGACCGCGGTCCGCAAGTACCAGGAGCAGTACCTCTACTGGTCGACC

121 G F G A G A V V A L A T Q P T D G V R K Y Q E Q Y L Y W S T

811 GACGGCGGGTTCACGTTACCGCCCTGCCCGACCCCGTCATCGTCAACACCGACGGTCCGCGCGCCACACGCCCGCGGAGATCGAGAAC

151 D G G F T F T A L P D P V I V N T D G R A A T T P A E I E N

15 901 GCCGAGTGGTTCGCGCACCCCAAGATCCACTGGGACACCGCCCGGAGAATGGGTCTGCGTCATCGGACGACTGCGGTACGCCGCGTTC

181 A E W F R D P K I H W D T A R G E W V C V I G R L R Y A A F

991 TACACCTCGCCGAACCTGCGCGACTGGACACTTCGCCGCAACTTCGACTACCCGAACACGCCCTCGCGCGCATCGAGTGCCCCGACCTG

211 Y T S P N L R D W T L R R N F D Y P N H A L G G I E C P D L

1081 TTCGAGATCACCGCAGACGACGGGACACGCCACTGGGTGCTCGCGCGCAGCATGGACGCCTACGGCATCGGCCCTCCCATGACGTACGCC

20 241 F E I T A D D G T R H W V L A A S M D A Y G I G L P M T Y A

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271 Y W T G T W D G E Q F H A D D L T P Q W L D W G W D W Y A A

ClaI Aval

1261 GTCACCTGGCCATCGATCGACGCGCCCGAGACCAAGCGCCTCGCCATCGCTGGATGAACAACTGGAAGTACGCCGACGCGACGTCCCC

25 301 V T W P S I D A P E T K R L A I A W M N N W K Y A A R D V P

1351 ACCGACGCATCCGACGGCTACAACGGGCAGAACTCGATCGTCCGCGAGCTGCGGCTCGCCGACAGCCTGGCGGCTGGTACACCTCCTG

331 T D A S D G Y N G Q N S I V R E L R L A R Q P G G W Y T L L

NcoI

1441 AGCACCCCGTGGCAGCGCTGACGAACTACGTACCGCCACCAACCACTCCCCGACCGGACCGTCGACGGCAGCGCGTCTGCCATGG

30 361 S T P V A A L T N Y V T A T T T L P D R T V D G S A V L P W

1531 AACGACGCGCATAAGAGATCGAGCTCGACATCGCCTGGGACACCGCGACGAACGTGCGCATCTCGGTGGCGCGCTCCCCGACGGAACC

391 N G R A Y E I E L D I A W D T A T N V G I S V G R S P D G T

1621 CGGCACACGAACATCGGCAAGTACGGAGCAGACCTGTACGTGACCGGAGGACCTCCGACCTCGCGGGTACTCGCTCGCCCCCTACTCG

421 R H T N I G K Y G A D L Y V D R G P S D L A G Y S L A P Y S

35 1711 CGAGCCGCGCGCCCATCGACCCCGCGCGCGATCCGTGCACCTGCGCATCTCGTCGACACCCAGAGCGTGAGGTCTTCGTCAACGCC

451 R A A A P I D P G A R S V H L R I L V D T Q S V E V F V N A
 1801 GGCCACACCGTGCTCTCCAGCAGGTCCACTTCGCCGAGGGCGACACGGGAATCTCGCTCTACACCGACGGCGGCCCGCACACTTCACC
 481 G H T V L S Q Q V H F A E G D T G I S L Y T D G G P A H F T
 SmaI
 5 1891 GGCATCGTCGTCGCGAGATTGGCCAGGCGATCTAGGCGATGCACACCACACCGCTCACCGAAGCCGCGCCCCGGGAGACGACGGCCGAC
 511 G I V V R E I G Q A I *
 1981 AATCGACACGTCCTCGTCGTT

6. A recombinant expression vector, carrying the levan fructotransferase
 10 gene of claim 3.

7. A recombinant expression vector as set forth in claim 6, wherein said
 expression vector is pUDFA81.

15 8. A novel transformant transformed with a recombinant expression
 vector as set forth in claim 6.

9. A novel organism *E. coli* JUD81 (KCTC 0877BP), which is prepared
 by transforming *E. coli* DH5 α with the expression vector pUDFA81 of claim 7.
 20

10. A process for producing difructose dianhydride IV from levan, in
 which a levan solution is subjected to reaction at 25-50°C for 3-10 hours in an
 acidic buffer of pH 3.0-7.0 in the presence of a levan fructotransferase.

25 11. The process as set forth in claim 10, wherein the reaction is carried
 out at 37°C.

12. The process as set forth in claim 10, wherein the acidic buffer is a

phosphate buffer of pH 5.8

13. The process as set forth in claim 10, wherein the levan solution has a levan concentration of 5-15 % (w/v).

5

14. The process as set forth in claim 10, wherein the levan fructotransferase has histidine residues at its C- or N-terminus.

15. A process of preparing levan fructotransferase, comprising the steps
10 of:

culturing a bacterial species anchoring a levan fructotransferase gene-carrying, expression plasmid;

harvesting and homogenizing the cells; and

isolating levan fructotransferase from the cell homogenate.

15

16. The process as set forth in claim 15, wherein the levan fructotransferase has histidine residues at its N- or C-terminus.

17. The process as set forth in claim 15, wherein the isolating step is
20 carried out using metal ion-affinity chromatography.

18. A process for producing difructose dianhydride IV from sucrose, comprising the steps of:

25 reacting a sugar solution at room temperature or lower in an acidic buffer of pH 3.0-7.0 in the presence of a levansucrase to produce levan;

purifying the levan from the sugar reaction mixture, partially or completely;

reacting a levan solution at 25-50°C for 3-10 hours in an acidic buffer of pH 3.0-7.0 in the presence of a levan fructotransferase to produce difructose dianhydride IV; and

isolating the difructose dianhydride IV from the levan reaction mixture.

5

19. The process as set forth in claim 18, wherein the levansucrase is derived from *Z. mobilis*.

20. The process as set forth in claim 18, wherein the sugar solution is
10 reacted at 0-15°C.

21. The process as set forth in claim 18, wherein the sugar solution has a sugar concentration of 10-30 % (w/v).

15 22. The process as set forth in claim 18, wherein the levan solution is reacted at 37°C.

23. The process as set forth in claim 18, wherein the acidic buffer for the sugar solution is an acetic acid buffer of pH 5.0 and the acidic buffer for the
20 levan solution is a phosphate buffer of pH 5.8

24. The process as set forth in claim 18, wherein the levan solution has a levan concentration of 5-15 % (w/v).

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(74) Agent: **LEE, Duckro**; 2nd Fl., Yeil Bldg., 700-19 Yorksam-dong, Kangnam-gu, Seoul 135-928 (KR).

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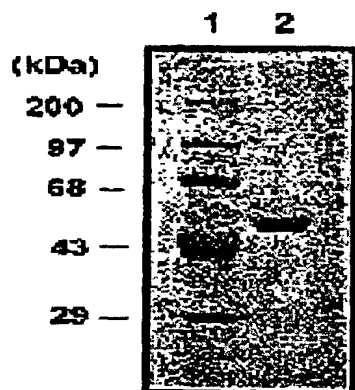
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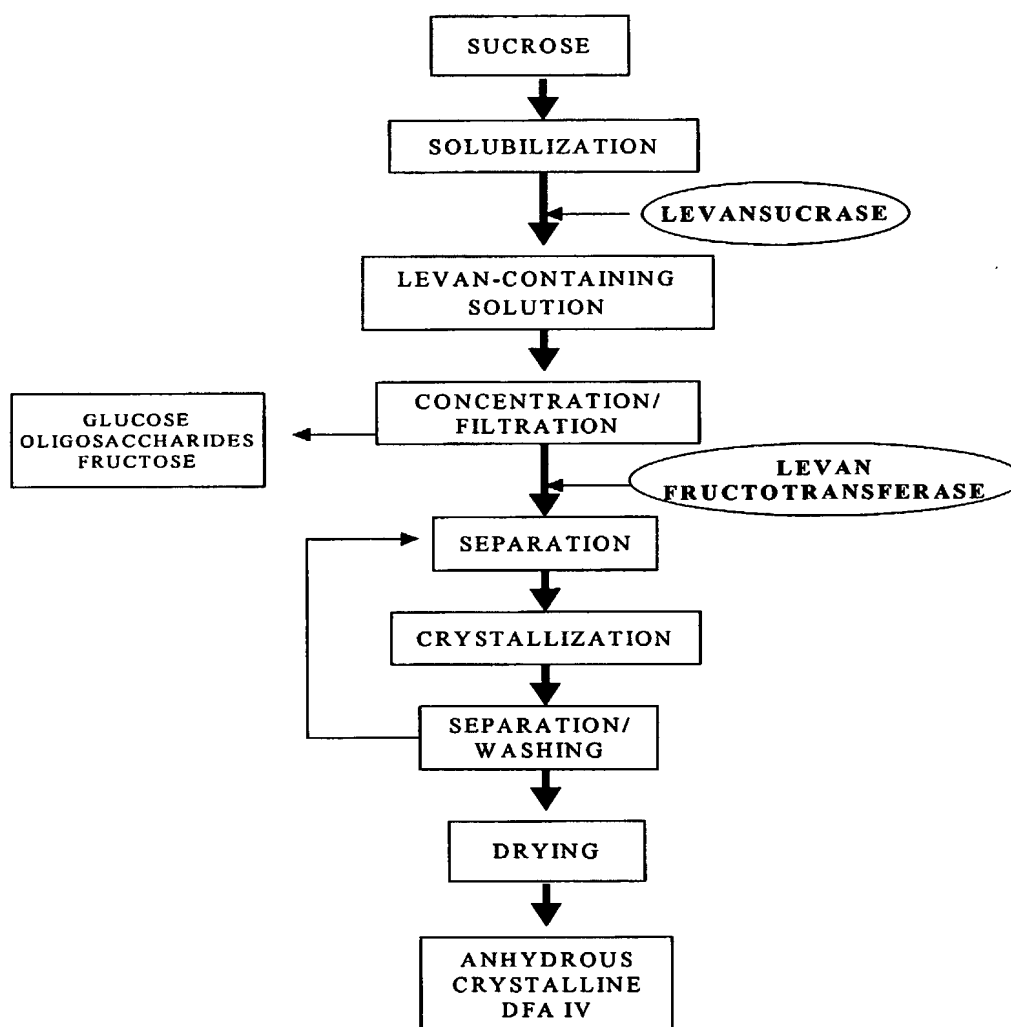
(54) Title: ENZYMATIC PRODUCTION OF DIFRUCTOSE DIANHYDRIDE IV FROM SUCROSE AND RELEVANT ENZYMES AND GENES CODING FOR THEM



(57) Abstract: Disclosed is the production of difructose dianhydride IV from sugar. A sugar solution is subjected to reaction at room temperature or lower in an acidic buffer of pH 3.0-7.0 in the presence of a levansucrase derived from *Z. mobilis* to produce levan. The levansucrase is prepared by culturing *E. coli* BL21 (DE3)/pEL12 (KCTC 8661), harvesting and homogenizing the cells, and isolating levansucrase from the cell homogenate. Levan is purified from the reaction solution and subjected to reaction at 25-50 °C for 3-10 hours in an acidic buffer of pH 3.0-7.0 in the presence of a levan fructotransferase to produce difructose dianhydride IV. The levan fructotransferase is obtained from *E. coli* JUD81 (KCTC 0877BP). Also, disclosed are a gene coding for the levan fructotransferase and an expression vector pUDAF81 carrying the gene.

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Fig. 1

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Fig. 2

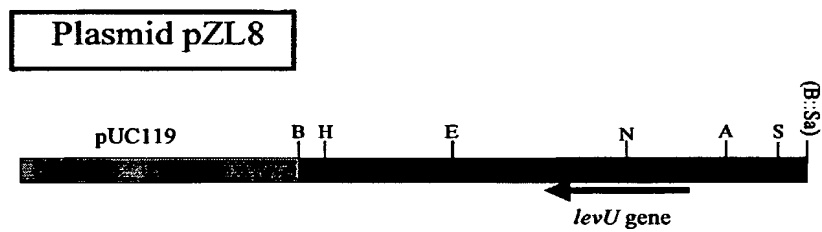


Fig. 3

[illegible]

Fig. 4

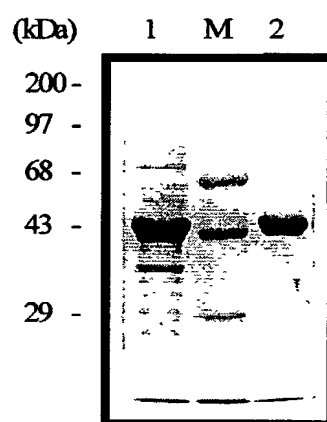
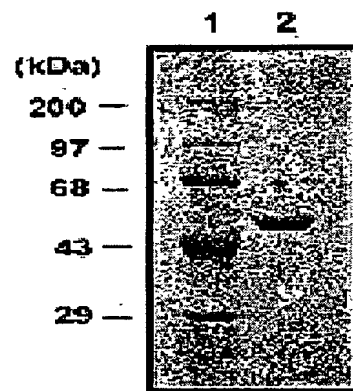


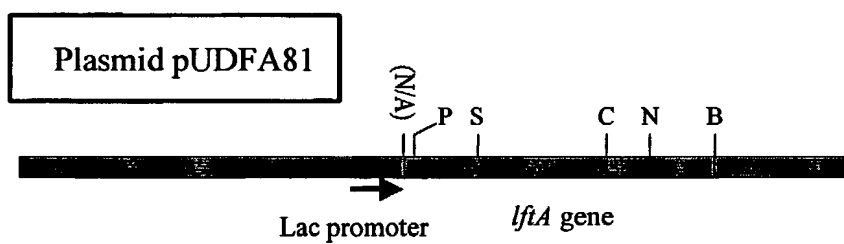
Fig. 5



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Fig. 7



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Fig. 8

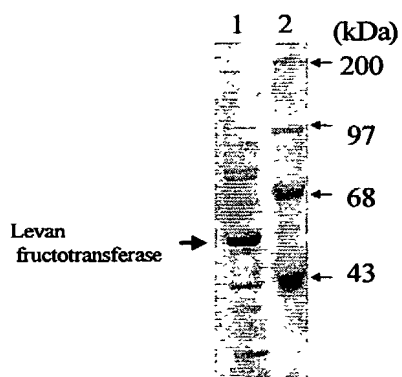
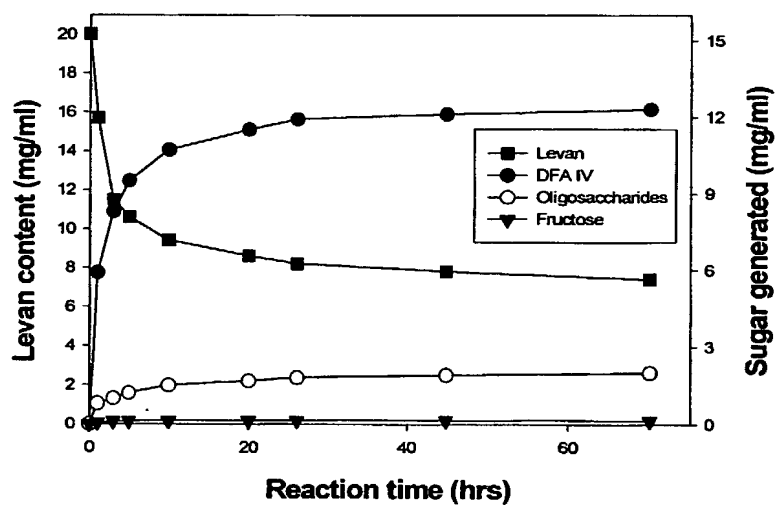


Fig. 9

Attorney Docket: 24679
Page 1 of 2

As a below-named inventor(s), I/we hereby declare that:

TRADEMARK My/Our residence(s), post office address(es) and citizenship(s) is/are as stated below next to my/our name(s).

I/We believe I/we am/are the original inventor, first and sole (if only one name is listed below) or the original, first and joint inventors (if plural names are listed below) of the subject matter which is claimed, and for which a patent is sought on the invention entitled:

ENZYMATIC PRODUCTION OF DIFRUCTOSE DIANHYDRIDE IV FROM SUCROSE AND RELEVANT ENZYMES AND GENES CODING FOR THEM

the specification of which: (check one)

[] is attached hereto.

[X] was filed on 19 October 2000, as Serial No. PCT/KR00/01183,
18 June 2001 09/868,328
 and was amended on _____ (if applicable).

We hereby state that we have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

We acknowledge the duty to disclose information which is material to the patentability of this application as defined by 37 CFR § 1.56.

We hereby claim foreign priority benefits under 35 U.S.C. § 119 of any foreign application(s) for patent or inventor's certificate listed below, and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

Prior Foreign Applications:

<u>1999-45302</u> (Application No.)	<u>KR</u> (Country)	<u>19 / Oct. / 1999</u> (Day/Month/Year Filed)	<u>Priority Claimed</u> [X] Yes [] No
<u> </u> (Application No.)	<u> </u> (Country)	<u> / / </u> (Day/Month/Year Filed)	[] Yes [] No
<u> </u> (Application No.)	<u> </u> (Country)	<u> / / </u> (Day/Month/Year Filed)	[] Yes [] No
<u> </u> (Application No.)	<u> </u> (Country)	<u> / / </u> (Day/Month/Year Filed)	[] Yes [] No

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020529

PATENT TRADEMARK OFFICE

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We hereby claim the benefit under 35 U.S.C. § 120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by 35 U.S.C. § 112, first paragraph, I/we acknowledge the duty to disclose material information as defined in 37 CFR § 1.56 which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

(U.S. Application Serial No.)	(U.S. Filing Date)	(Status--patented, pending, abandoned)
2,541,000	12-1-40	Patented
2,541,001	12-1-40	Patented
2,541,002	12-1-40	Patented
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2,541,093	12-1-40	Patented

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2,342,000	12-1-35	patented
2,342,001	12-1-35	patented
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2,342,003	12-1-35	patented
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2,342,092	12-1-35	patented
2,342,093	12-	

DECLARATION FOR PATENT APPLICATION

Attorney Docket: 24679
Page 2 of 2

I/We hereby claim the benefit under 35 U.S.C 119(e) of any United States provisional application(s) listed below:

(Application Number(s))

Filing Date

We hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. § 1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

1-00
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Date AUGUST 10, 2001

Residence: 2-1101, Shindonga Apt., Yongjeon-dong, Dong-qu, Taejon, 300-766 REPUBLIC OF KOREA

Country of Citizenship: REPUBLIC OF KOREA

Post Office Address: same as residence

4-00
Full name of fourth inventor: Eunja RYU

Inventor's Signature

Date AUGUST 10, 2001

Residence: 107-1302, Hanbit APT., #99, Oun-dong, Yusong-qu, Taejon, 305-755 REPUBLIC OF KOREA

Country of Citizenship: REPUBLIC OF KOREA

Post Office Address: same as residence

5-00
Full name of fifth inventor: Yongbok LEE

Inventor's Signature

Date AUGUST 10, 2001

Residence: 101-1703, Sangyong Apt., Ilgok-dong, Buk-qu, Gwangju, 500-160 REPUBLIC OF KOREA

Country of Citizenship: REPUBLIC OF KOREA

Post Office Address: same as residence



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SEQUENCE LISTING

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KIM, Chulho
RYU, Eunja
LEE, Yongbok

<120> Enzymatic Production of Diffructose Dianhydride IV from sucrose and relevant enzymes and genes coding for them

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<141> 2001-06-18

<150> PCT/KR00/01183

<151> 2000-10-19

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Phe Glu Ile Thr Ala Asp Asp Gly Thr Arg His Trp Val Leu Ala Ala
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Trp Ser Val Ile Phe Ala Leu Val Ala Asp Arg Thr Lys Tyr Gly Trp
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Ala Arg Thr Glu Trp Lys Leu Leu Pro Pro Leu Val Thr Ala Phe Gly
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SEQUENCE LISTING

<110> Korea Research Institute of Bioscience and Biotechnology
Realbiotech, Ltd.
RHEE, Sangki
SONG, Kibang
KIM, Chulho
RYU, Eunja
LEE, Yongbok

<120> ENZYMATIC PRODUCTION OF DIFRUCTOSE DIANHYDRIDE IV FROM SUCROSE
AND RELEVANT ENZYMES AND GENES CODING FOR THEM

<130> YL001019BPCT

<150> 1999-45302

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WO 01/29185

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SEQUENCE LISTING

<110> Korea Research Institute of Bioscience and Biotechnology
Realbiotech, Ltd.
RHEE, Sangki
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<120> ENZYMATIC PRODUCTION OF DIFRUCTOSE DIANHYDRIDE IV FROM SUCROSE
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Phe Asp Tyr Pro Asn His Ala Leu Gly Gly Ile Glu Cys Pro Asp Leu		
225	230	235 240
Phe Glu Ile Thr Ala Asp Asp Gly Thr Arg His Trp Val Leu Ala Ala		
245	250	255
Ser Met Asp Ala Tyr Gly Ile Gly Leu Pro Met Thr Tyr Ala Tyr Trp		
260	265	270
Thr Gly Thr Trp Asp Gly Glu Gln Phe His Ala Asp Asp Leu Thr Pro		
275	280	285
Gln Trp Leu Asp Trp Gly Trp Asp Trp Tyr Ala Ala Val Thr Trp Pro		
290	295	300
Ser Ile Asp Ala Pro Glu Thr Lys Arg Leu Ala Ile Ala Trp Met Asn		
305	310	315 320
Asn Trp Lys Tyr Ala Ala Arg Asp Val Pro Thr Asp Ala Ser Asp Gly		
325	330	335
Tyr Asn Gly Gln Asn Ser Ile Val Arg Glu Leu Arg Leu Ala Arg Gln		
340	345	350
Pro Gly Gly Trp Tyr Thr Leu Leu Ser Thr Pro Val Ala Ala Leu Thr		

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355	360	365
Asn Tyr Val Thr Ala Thr Thr Thr Leu Pro Asp Arg Thr Val Asp Gly		
370	375	380
Ser Ala Val Leu Pro Trp Asn Gly Arg Ala Tyr Glu Ile Glu Leu Asp		
385	390	400
Ile Ala Trp Asp Thr Ala Thr Asn Val Gly Ile Ser Val Gly Arg Ser		
405	410	415
Pro Asp Gly Thr Arg His Thr Asn Ile Gly Lys Tyr Gly Ala Asp Leu		
420	425	430
Tyr Val Asp Arg Gly Pro Ser Asp Leu Ala Gly Tyr Ser Leu Ala Pro		
435	440	445
Tyr Ser Arg Ala Ala Ala Pro Ile Asp Pro Gly Ala Arg Ser Val His		
450	455	460
Leu Arg Ile Leu Val Asp Thr Gln Ser Val Glu Val Phe Val Asn Ala		
465	470	475
Gly His Thr Val Leu Ser Gln Gln Val His Phe Ala Glu Gly Asp Thr		
485	490	495
Gly Ile Ser Leu Tyr Thr Asp Gly Gly Pro Ala His Phe Thr Gly Ile		
500	505	510
Val Val Arg Glu Ile Gly Gln Ala Ile		
515	520	